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**14. ABSTRACT**

We hypothesized that (1) prostate cancer cells that express high levels of cyclooxygenase-2 (COX-2) and prostaglandin E2(PGE2) display enhanced bone targeting and (2) the level of expression of COX-2 and PGE2 in established bone metastases determines the overall bone response, with lower vs. higher levels inducing osteoblastic vs. osteolytic responses, respectively. We utilized two human prostate cancer cell lines (MDA-PCa-2B that expresses low levels of COX-2 and PGE2 and produces osteoblastic lesions vs. PC-3 that expresses high levels COX-2/PGE2 and induces osteolytic mets). We demonstrated that (1) low levels of PGE2 stimulate preosteoblast cell growth, differentiation and Wnt signaling (2) Forced overexpression of COX-2 in MDA-PCa-2b cells induces the Wnt antagonist DKK-1 (3) PGE2 addition to PC-3 cells stimulates Dkk-1 (4) Forced overexpression of COX-2 in MDA-PCa-2B cells inhibits preosteoblastic cell growth in co-culture and, finally, (5) Treatment with a COX-2 inhibitor reduced PC-3 metastatic lesons in vivo after intracardiac inoculation. Over the next several weeks we will analyze bone metastatic lesions to determine overall bone response in vivo from the PC-3 experiments and from tibia of mice inoculated with wild-type vs. COX-2 overexpressing MDA-PCa-2b cells. These studies confirmed our hypothesis that levels of COX-2/PGE2 expression in prostate cancer cells modulates both bone targeting and bone response.

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rostate cancer, bone metastases, COX-2, PGE2, osteoblastic, osteolytic

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## INTRODUCTION

The overall purpose of these studies was to investigate our hypothesis that dose-dependent expression of COX-2/PGE2/IL-6 by prostate cancer cells influences both *bone targeting* and the *bone response* (osteoblastic vs. osteolytic) once prostate cancer cells reside in the bone microenvironment. Specifically, we hypothesized that low levels of COX-2/PGE2/IL-6 expression favor an osteoblastic bone response whereas higher expression levels by prostate cancer cells produce osteolytic lesions. We also hypothesized that high levels of COX-2/PGE2 expression by prostate cancer cells promoted bone-targeting, as the initial phases of prostate cancer bone metastases involve osteoclastic bone resorption (1). In order to prove these hypotheses, we proposed a series of *in vitro* and *in vivo* experiments, utilizing human prostate cancer cell lines that differentially express COX-2/PGE2, have varying ability to target bone in animal model systems and induce different bone reactions. Our lab and others have also demonstrated differential expression by human prostate cancer cell lines of inhibitors of Wnt signaling (i.e. Dkk-1) (1, 2). The Wnt signaling pathway is known to regulate osteoblastic differentiation and we have further investigated the interrelationships between Wnt inhibitor expression/COX-2/PGE2 and the induction of an osteoblastic vs. osteolytic bone reaction by human prostate cancer cell lines.

## BODY

During the three years of funding, we investigated the effects of COX-2 and its major product, PGE2, on the regulation of prostate cancer bone metastases. Our hypothesis is that dose-dependent expression of COX-2/PGE2 by prostate cancer cells influences both bone targeting and the bone response (osteoblastic vs. osteolytic) once prostate cancer cells reside in the bone microenvironment. We postulated, specifically, that low levels of COX-2/PGE2 expression/secretion favor an osteoblastic bone response (i.e. MDA-PCa-2b cells) whereas higher expression/secretion levels by prostate cancer cells (i.e. PC-3 cells) produce osteolytic lesions. We have published data demonstrating that PGE2 (at doses > 1μM) and IL-6 promote osteoclastogenesis via the OPG/RANK/RANKL system (3). We determined the effects of COX-2 and PGE2 on the growth of MC3T3 cells, which are mouse pre-osteoblasts. In light of recent reports indicating that the Wnt and BMP signaling pathways are key mediators of osteoblast differentiation (2), we expanded our original studies to include analysis of the dose-dependent effects of COX-2/$PGE_2$ on components of the Wnt signaling pathway in MC3T3 mouse preosteoblast cells. Utilizing co-cultures of prostate cancer cells and MC3T3 cells, we determined the effects of altering COX-2/$PGE_2$ expression/secretion by the cancer cells on the growth and Wnt signaling activity in the preosteoblast cells. Finally, during the last year of the project, we conducted *in vivo* experiments to determine the dose-dependent effects of COX-2/$PGE_2$ expression on bone targeting and bone response in an animal model system.

### 1. Relative expression levels of COX-2 in PCa cell lines.

We performed Western blots to determine the relative basal expression levels of COX-2 in human prostate cancer cell lines. Both PC-3 and its more aggressive subline, PC-3 ML have high basal COX-2 expression levels and both of these lines can target bone in animal models and induce an osteolytic reaction in bone. In contrast, MDA-PCa-2b cells do not target bone significantly or induce metastases elsewhere when inoculated via the intracardiac route in nude mice. When directly inoculated into bone, MDA-PCa-2b cells induce an osteoblastic bone reaction and this corresponds to low basal COX-2 expression (Fig 1, below)

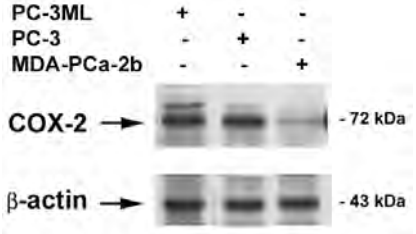


***Fig. 1*: Relative COX-2 expression levels in various PCa cell lines.**

**2. $PGE_2$ exerts dose-dependent effects on MC3T3 pre-osteoblast growth and differentiation.**

PGE2, at low doses (< 1μM) stimulated MC3T3 growth, and slightly increased their differentiation. At higher doses (> 1μM), PGE2 inhibited both the growth and differentiation of the bone cells. These effects were dose-dependent with maximal inhibitory effects demonstrated at the highest dose of 10μM (Fig.2A&B). We further examined the effect of PGE2 on Wnt signaling utilizing a T-cell factor luciferase (Tcf-Luc) reporter assay. As shown in Fig.2C, while low doses of PGE2 slightly increased Tcf-Luc activity, high doses of PGE2 significantly inhibited this measure of Wnt signaling activity.

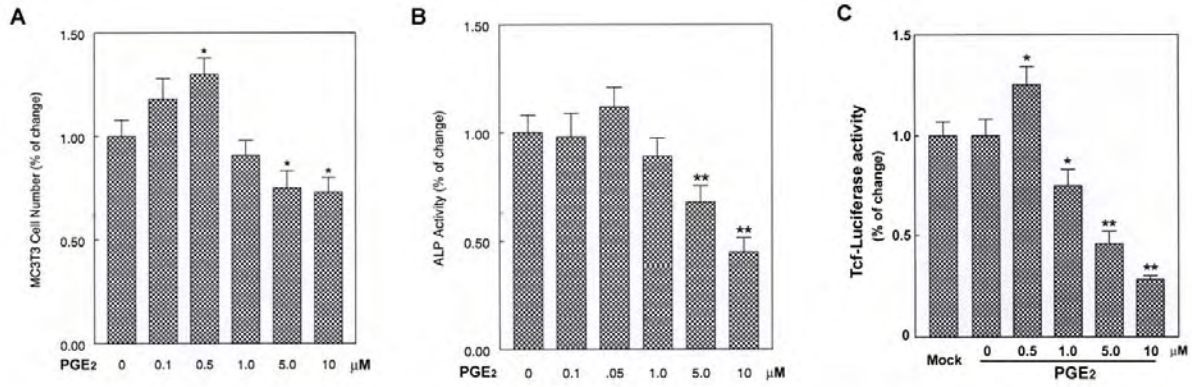


***Fig. 2. Direct effects of PGE2 on preosteoblast cells.*** *(A) Cell growth*, MC3T3 cells were cultured in serum-free medium and treated +/- various doses of PGE2, as indicated, for 7 days. Cell numbers were counted using a hemocytometer. *(B) Differentiation*, MC3T3 cells were cultured in serum-free medium and treated +/- various doses of PGE2, as indicated, for 14 days. Alkaline phosphatase (ALP) activity in the lysates was assayed by the hydrolysis of *p*-nitrophenyl phosphate (Sigma) to *p*-nitrophenol. Absorbance was determined at 405 nm and compared with a *p*-nitrophenol (Sigma) standard titration curve. ALP activity was normalized to total protein content. *(C) Tcf-Luciferase reporter activity.* Cells were co-transfected with the Tcf reporter plasmid (or empty vector as mock control) and the β-galactosidase expression vector and treated with either vehicle or various doses of PGE2 for 24h. The resulting Tcf-luciferase activities were normalized to protein concentrations and β-galactosidase activity. Data are expressed as fold-induction compared with the vehicle control (100%), and represent the means ± SE from three separate determinations. * $p < 0.05$, ** $p < 0.01$.

**3. COX-2 and PGE2 regulate the production of natural inhibitors of Wnt signaling in prostate cancer cells.** Osteoblast development is closely regulated by Wnt signaling (2). Naturally occurring Wnt inhibitors such as sFRP and DKK play an important role in the modulation of Wnt signaling in bone. MC3T3 pre-osteoblast cells do not secrete significant amounts of either inhibitor. We demonstrate that PC-3 and PC-3ML human prostate cancer cells (which induce an osteolytic reaction *in vivo*) secrete high amounts of both Dkk-1 and sFRP-1. In contrast, MDA-PCa-2b human prostate cancer cells (which induce an osteoblastic bone reaction *in vivo*) do not secrete significant amounts of either inhibitor (Fig. 3). These data strongly support previous findings from our laboratory as well as other investigators indicating that the development and progression of prostate cancer metastases in the bone environment are modulated by interactions between prostate cancer epithelium and bone cells via components of the Wnt signaling system (3).

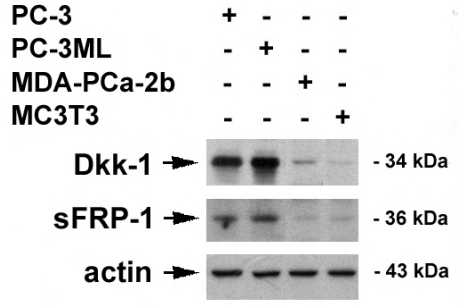


***Fig. 3. Protein expression of Dkk-1 and sFRP-1 in PCa and MC3T3 preosteoblast cell lines assayed by Western blotting.*** Cells were lysed and subjected to Western Blot analysis. Protein content was assayed and 30μg total protein was loaded in each lane.

*a) Co-expression of COX-2 and DKK-1 in MDA-PCa-2b cells with forced expression of COX-2 protein.* We established several sub-lines of MDA-PCa-2b that stably express COX-2 and examined the expression of the, Dkk-1. As shown in Fig.4, the harvested clones that over-expressed COX-2 also expressed significant amounts of Dkk-1 (Fig.4A) indicating that COX-2 may regulate the production of this Wnt inhibitor, thereby modulating Wnt activity in a paracrine fashion in the bone microenvironment. These data may explain the observations that PC-3 cells express both COX-2 and high levels of Dkk-1, and induce osteolytic lesions in nude mice, whereas MDA-PCa-2b cells, which induce an osteoblastic bone response, express undetectable amounts of COX-2 and low levels of Dkk-1.

*b) Modulation of Dkk-1 expression by COX-2 inhibitors and PGE2 addition.* We examined the expression of Dkk-1 in mock- and COX-2-transfected MDA-PCa-2b cells +/- various doses of PGE2, and in PC-3 cells +/- meloxicam, a selective COX-2 inhibitor. As shown in Fig.4B, Dkk-1 expression was increased by PGE2 and decreased by a COX-2 inhibitor in MDA-PCA-2b and PC-3 cell lines, respectively.

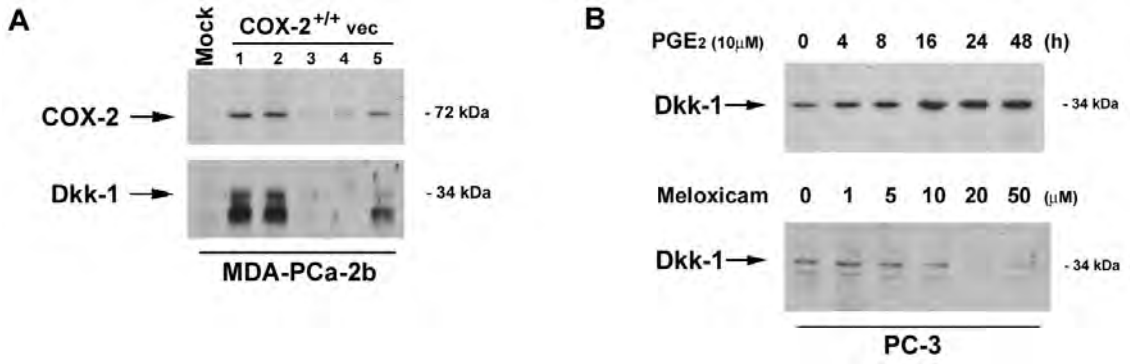


***Fig. 4*. COX-2 and PGE2 increase Dkk-1 protein expression in human prostate cancer cell lines..** *(A) Forced expression of COX-2 increases Dkk-1 and sFRP-1 protein levels in MDA-PCa-2b cells*. MDA-PCa-2b cells were stably transfected with the COX-2 expression vector. After selection with G418, the resultant clones were harvested and characterized by Western blot analysis. (B) *Effects of PGE2 and Meloxicam on Dkk-1 expression in PC-3 cells* assayed by Western blot analysis.

**4. Forced expression of COX-2 in MDA-PCa-2b cells suppressed MC3T3 preosteoblast cell growth and differentiation via inhibition of Wnt activity.**

To further determine the effects of COX-2/PGE2 on PCa-induced bone metastases, we utilized an *in vitro* co-culture system in which MC3T3 preosteoblast cells were plated on the bottom of a 12-well cluster plate and MDA-PCa-2b cells were plated in the inserts containing 0.4μM holes for communication. Growth rates, cell differentiation (assayed by alkaline phosphotase [ALP] activity) and Wnt signaling activity (assayed by Tcf-luc reporter assay) of the bone cells were determined. As demonstrated in Fig.5, we observed a small increase in the all three parameters when bone cells were co-cultured with mock-transfected MDA-PCa-2b cells. However, significant inhibition of those parameters was observed when the same bone cells were co-cultured with an MDA-PCa-2b subline with forced expression of COX-2 protein. These data are consistent with our previous findings that COX-2 increases the production of Dkk-1 and sFRP-1, two Wnt inhibitors, which in turn suppresses Wnt activity in preosteoblast cells, thereby inhibiting both their growth and differentiation.

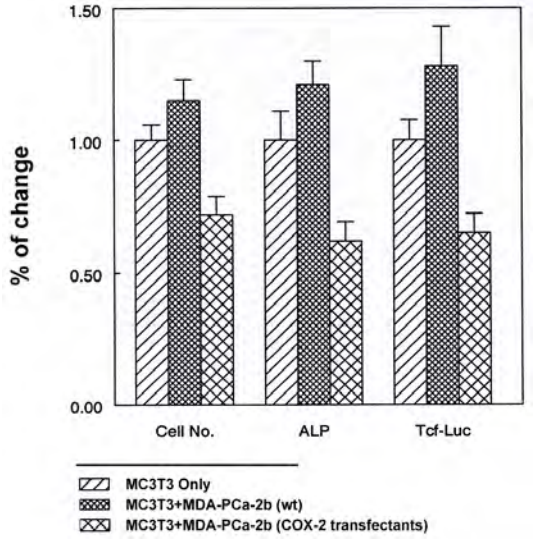


***Fig. 5. Forced expression of COX-2 in MDA-PCa-2b cells inhibits preosteoblastic cell growth, differentiation and Tcf-luciferase reporter activity in co-culture.*** MC3T3 cells were grown alone or co-cultured with either mock-transfected MDA-PCa-2b cells, or COX-2 transfectants for 7d. Cell numbers and ALP activity were assayed. Wnt signaling activity was determined by Tcf-Luciferase reporter assay and the results were normalized to protein content and β-gal activity. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

**5. Treatment with a COX-2 inhibitor (NS398) reduced PC-3 metastatic lesions *in vivo* after intracardiac inoculation.**

We generated a PC-3 subline (PC-3-Luc subline) by transfection of PC-3 cells with a construct expressing firefly luciferase (Luc). We then performed intracardiac inoculation of the cells (PC-3-Luc) into 22 nude mice. Two days after the inoculation, the 20 mice (2 mice died shortly after intracardiac inoculation) were randomized to receive either vehicle (control) of NS398, a selective COX-2 inhibitor, given intraperitoneally tri-weekly at a dosage of 3mg/kg body weight for 8 weeks. Treated (n=10) and control (vehicle) animals (n=10) were examined with *in vivo* bioluminescence image (BLI) to determine the extent of metastases. All mice (10 out of 10) treated with vehicle only demonstrated detectable BLI intensity. Five out of 10 mice in control group had distant metastases as measured by BLI. In contrast, 4 out of 10 mice in NS398-treated group showed no detectable BLI activity at all, and only 1 out of 10 mice developed distant metastases in the NS398-treated group. Fig. 6A demonstrates representative images of 3 pairs of mice from the control and NS398-treated mice groups. A quantitative comparison of total and local (derived from total and local metastatic areas) BLI intensity between control and NS398-treat mice is shown in Fig.6B. We are now performing histology studies to confirm the findings from BLI assay, and to determine whether the bone lesions are osteoblastic or osteolytic.

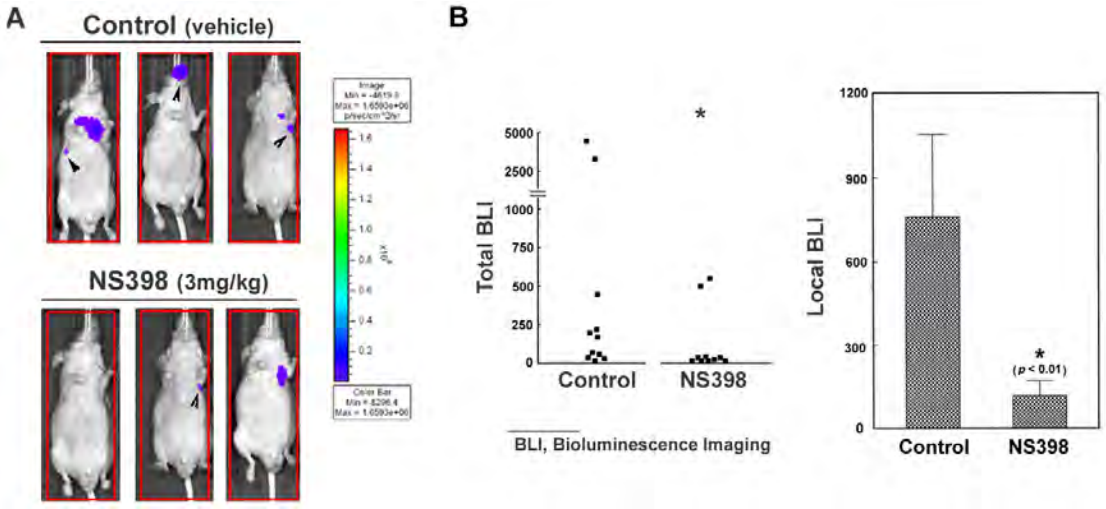


***Fig. 6.*** ***NS398 suppresses PC-3 cell metastasis in vivo.*** PC-3-Luc cells ($2x10^6$) in DMEM were injected into the cardiac left ventricle of 4- to 6-week old, male, athymic nude mice. Positive intra-cardiac injections were confirmed by whole body bioluminescence, and mice were randomized into 2 groups ($n$ = 10/group). *In vivo* bioluminescent imaging was performed monthly with an IVIS-200 imaging system (Xenogen Corp.). Fifteen minutes prior to imaging, mice were given 150 mg/kg luciferin by i.p. injection. Images were collected and analyzed with Living Image software (Xenogen Corp.), and the region of interest included the femur/tibia region of the hind limbs. *A)* Representative images of 3 pairs of mice from the control and NS398-treated mice group. *B)* Quantitative comparison of total and local (derived from total and local metastatic areas) BLI intensity between control and NS398-treat mice. $p < 0.01$ vs. control. Arrow heads indicate the areas of distant metastases.

**6. Intratibial injection of wt-MDA-PCa-2b vs. MDA-PCa-2b$^{cox2+/+}$ cells to determine whether forced COX-2 expression induces osteolytic bone response.**

In our final set of experiments, we injected wt vs. COX-$2^{+/+}$ MDA-PCa-2b cells intratibially into nude mice (n=6 in each group). The inoculations were carried out two months ago. As of the date of this report, two mice died (one from each group) leaving n=5 in each group. We plan on sacrificing the mice in another 4 weeks and analyzing their bones for tumor burder as well as osteoblastic vs osteolytic responses. We hypothesize that forced overexpression of COX-2 will induce an osteolytic response in contrast the osteoblastic response normally observed when intratibial inoculation is carried out with wt-MDA-PCa-2b cells which are have low COX-2 expression levels.

## KEY RESEARCH ACCOMPLISHMENTS

1. Determination of dose-dependent effects of PGE2 on preosteoblastic cell growth, differentiation and Wnt signaling with confirmation of our hypothesis that low doses of PGE2 stimulate preosteoblastic bone cell growth and differentiation whereas high PGE2 doses inhibit these activities via effects on Wnt signaling in the bone cells.

2. Establishment of a correlation between osteoblastic vs. osteolytic effects of various human prostate cancer cells and their expression levels of COX-2/PGE2 and Wnt inhibitors.

3. Characterization of our newly established sublines of the MDA-PCa-2b human prostate cancer cell lines that were stably transfected to overexpress COX-2/PGE2 with regards to its expression of the Wnt inhibitor Dkk-1 and the effect of this forced expression on preosteoblastic cell growth, differentiation and Wnt signaling in co-culture.

4. Transfection of PC-3 cells with a luciferase construct followed by intracardiac injection into nude mice. PC-3 metastases were then tracked using in vivo bioluminescence.

5. Demonstration that a COX-2 inhibitor, NS-398, reduced *in vivo* metastases of PC-3-luciferase-tagged cells.

## REPORTABLE OUTCOMES

### ***Manuscripts***

1. Liu, X.H., Kirschenbaum, A., Yao, S., and Levine, A.C. Interactive Effects of Interleukin-6 and Prostaglandin E2 on Osteoclastogenesis via the OPG/RANKL/RANK System. *Ann. N.Y. Acad.Sci.* 1068:225-233, 2006.

2. Liu, X.H., Kirschenbaum, A., Yao, S., Aaronson, S.A., Liu, G. and Levine, A.C. Androgen-induced Wnt signaling activation in preosteoblasts promotes the growth of MDA-PCa-2b human prostate cancer cells. *Cancer Res.* 67 (12):5747-53, 2007.

3. Liu, X.H., Kirschenbaum, A., Yao, S., and Levine, A.C. Androgens promote preosteoblast differentiation via activation of the canonical Wnt signaling pathway. *Ann. NY Acad. Sci*. 1116:423-431, 2007.

### ***Abstracts***

1. Liu, X.H, Kirschenbaum, A., Yao, S., and Levine, A.C. Androgen-induced Wnt signaling activation in osteoblasts is critical for prostate cancer cell growth in the bone microenvironment. Annual Meeting of American Association of Cancer Research (AACR), Washington D.C., April 2006.
2. Levine, A.C., Kirschenbaum, A., Yao, S., Liu, X.H. Androgens differentially regulate Wnt signaling and osteoblast differentiation in stage-specific bone-related cell lines. 88$^{th}$ Annual Meeting of the Endocrine Society, Boston, MA, June 2006.
3. Liu, X.H., Kirschenbaum, A., Yao, S., Liu, G., Aaronson, S.A. and Levine, A.C. Androgen-induced Wnt signaling in preosteoblasts promotes the growth of MDA-PCA-2B human prostate cancer cells in co-culture. 89$^{th}$ Annual Meeting of the Endocrine Society, Toronto, CN, June 2007.
4. Liu, X.H., Yao, S., Kirschenbaum, A. and Levine, A.C. Effects of cyclooxygenase-2 and prostaglandin E2 on prostate cancer bone metastases. First IMPACT meeting of DOD grantees, Atlanta, GA, September 2007.
5. Weinstein, B.M., Liu, X.-H., Yao, S., Kirschenbaum, A., and Levine, A.C. Dose-dependent effects of $PGE_2$ on osteoblasts: implications for osteoporosis treatment. Endocrine Society 90$^{th}$ Annual Meeting to be held in San Diego, CA, June 2008 (Abstract submitted).

## CONCLUSIONS

We proved out hypotheses that dose-dependent effects of COX-2/PGE2 modulate both bone targeting and bone reaction in metastatic prostate cancer. Specifically, we demonstrated that increased COX-2/PGE2 by prostate cancer cells promote bone targeting *in vivo*. Once prostate cancer cells metastasize and reside in the bone microenvironment, low levels of PGE2 secretion by PCa cells promote osteoblastogenesis whereas high levels inhibit osteoblastogenesis, via dose-dependent effects on Wnt signaling in osteoblasts. These data have significant clinical implications in the prevention and treatment of prostate cancer bone metastases.

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4. Hall CL, Bafico A, Dai J, Aaronson SA, Keller ET. Prostate cancer cells promote osteoblastic bone metastases through Wnts. *Cancer Res*. 65:7554-7560, 2005.

# Interactive Effect of Interleukin-6 and Prostaglandin $E_2$ on Osteoclastogenesis via the OPG/RANKL/RANK System

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**ABSTRACT: The OPG/RANKL/RANK system regulates osteoclastogenesis. Both cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) and interleukin-6 (IL-6) are reported to induce osteoclast differentiation. The mechanisms underlying these signaling pathways on the OPG/RANKL/RANK system are not fully understood. We herein demonstrate that COX-2 and PGE2 stimulated osteoclastogenesis through inhibition of OPG secretion, stimulation of RANKL production by osteoblasts, and upregulation of RANK expression in osteoclasts. PGE2 also stimulated IL-6 production, and IL-6, in turn, increased PGE2 secretion, COX-2, and $EP_4$/$EP_2$ expression in bone cells. These findings provide evidence of interactive effect of PGE2 and IL-6 signaling pathways in osteoclastogenesis via effect on the OPG/RANKL/RANK system.**

**KEYWORDS: COX-2; PGE2; IL-6; OPG/RANKL/RANK system; osteoclastogenesis**

## INTRODUCTION

The cellular interactions of osteoblasts and osteoclasts determine the type and extent of bone remodeling. These interactions are mediated by receptor activator of NFκB (RANK) on the osteoclast surface and a balance in RANK ligand (RANKL) and osteoprotegerin (OPG) production by osteoblasts, i.e., the RANKL/OPG/RANK system.[1] COX-2, the highly inducible enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, and PGE2, a major eicosanoid product of the COX-2-catalyzed reaction, play important roles in osteoclast formation *in vitro* and *in vivo*.[2] COX-2 and PGE2 have also

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been demonstrated to be critical regulators of osteoblast differentiation and essential elements in bone repair.[3] These data indicate that COX-2 and PGE2 are involved in both osteoclast activation and osteoblastic bone formation. IL-6 is a major proinflammatory cytokine that regulates osteoclastic bone resorption and has been implicated in the pathogenesis of several bone diseases.[4] IL-6 has also been reported to stimulate bone formation.[5] Although interactions between the COX-2/PGE2 and IL-6 systems have been described, the mechanisms underlying these cooperative signaling pathways and the possible involvement of the OPG/RANKL/RANK system in bone remodeling have not been fully elucidated. In the present study, we demonstrate interactive roles of COX-2/PGE2 and IL-6 signaling pathways in the regulation of osteoclastogenesis via the OPG/RANKL/RANK system.

## MATERIALS AND METHODS

The osteoblastic MC3T3 cell line was co-cultured with osteoclastic precursor cells (RAW264.7) in DMEM containing 10% FBS, and treated with or without various factors. Protein expression of OPG, COX-2, RANK, and subtypes of EP receptor was determined by using Western blotting. The secretion of soluble factors, i.e., IL-6, PGE2, OPG, and RANKL was measured by enzyme-linked immuno-sorbent assay (ELISA). The expression of subtypes of EP receptor mRNA was evaluated by using polymerase chain reaction (PCR).

## RESULTS AND DISCUSSION

### *Effects of PGE2 and IL-6 on Osteoblast Growth and Osteoclast Formation*

We initially determined the effects of PGE2 and IL-6 on osteoblast growth and osteoclast differentiation. As shown in FIGURE 1A, PGE2 and IL-6 stimulated MC3T3 cell growth. In contrast, two COX-2 inhibitors, NS398 and meloxicam, decreased osteoblast cell growth, which could be reversed by the addition of exogenous PGE2, suggesting a COX-2-dependent mechanism. The growth of osteoblasts was also stimulated by $PGE_1$-alcohol, a selective $EP_4$ receptor agonist, but not Butaprost, a selective $EP_2$ receptor agonist, indicating that PGE2-stimulated osteoblast cell proliferation is mediated by the $EP_4$ receptor subtype. FIGURE 1B demonstrates a significant increase in RAW264.7 cell differentiation induced by PGE2 and IL-6 when the cells were grown in co-culture with MC3T3 cells, compared to RAW264.7 cells grown alone.

### *Effects of PGE2 and IL-6 on RANKL and OPG Production and RANK Expression*

Our initial studies suggested that the effects of PGE2 and IL-6 on osteoclasts are mediated by the RANK/RANKL/OPG system. Next we determined

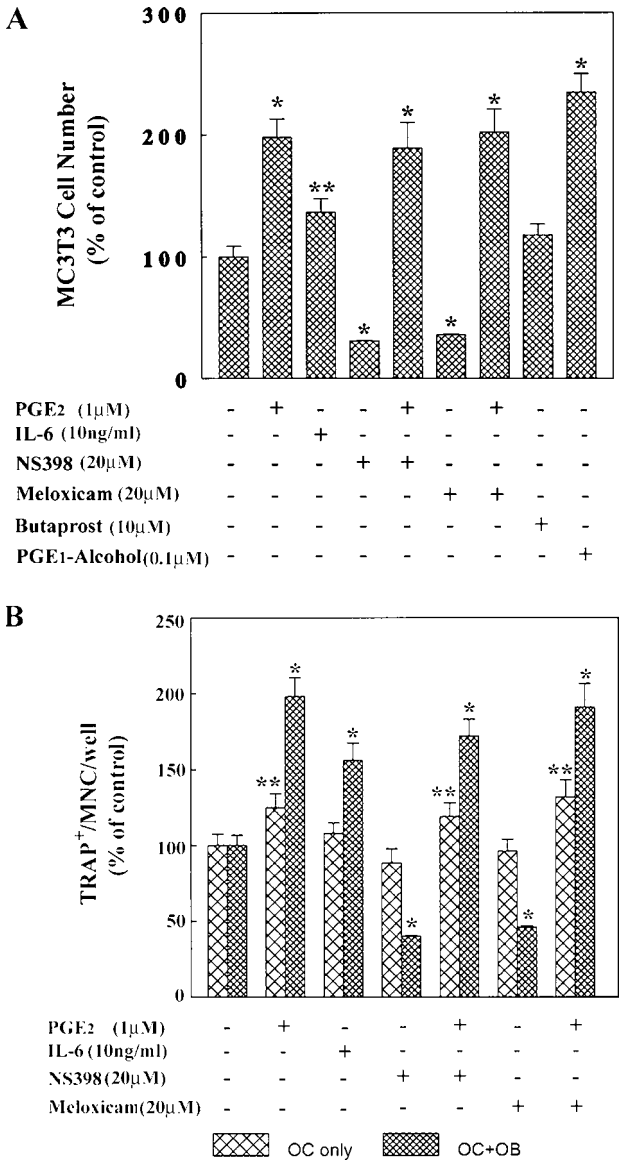


**FIGURE 1.** Effects of PGE2 and IL-6 on osteoblast growth and osteoclast formation. (**A**) Effects of PGE2 and IL-6 on osteoblast growth. MC3T3 cells were cultured in serum-free medium (SFM) and subjected to various treatments, as indicated, for 3 days. The number of living cells was counted. (**B**) Effects of PGE2, IL-6, and COX-2 inhibitors on osteoclast formation in the presence or absence of osteoblasts. RAW264.7 cells were cultured, either alone or co-cultured with MC3T3 cells, in SFM and treated with various of treatments for 3 days. The number of osteoclast-like cells was determined by counting TRAP-positive multinucleated cells (MNCs) with three or more nuclei. The data represent means ± SEM from three separate experiments. $^{**}P < 0.05$, $^{*}P < 0.01$.

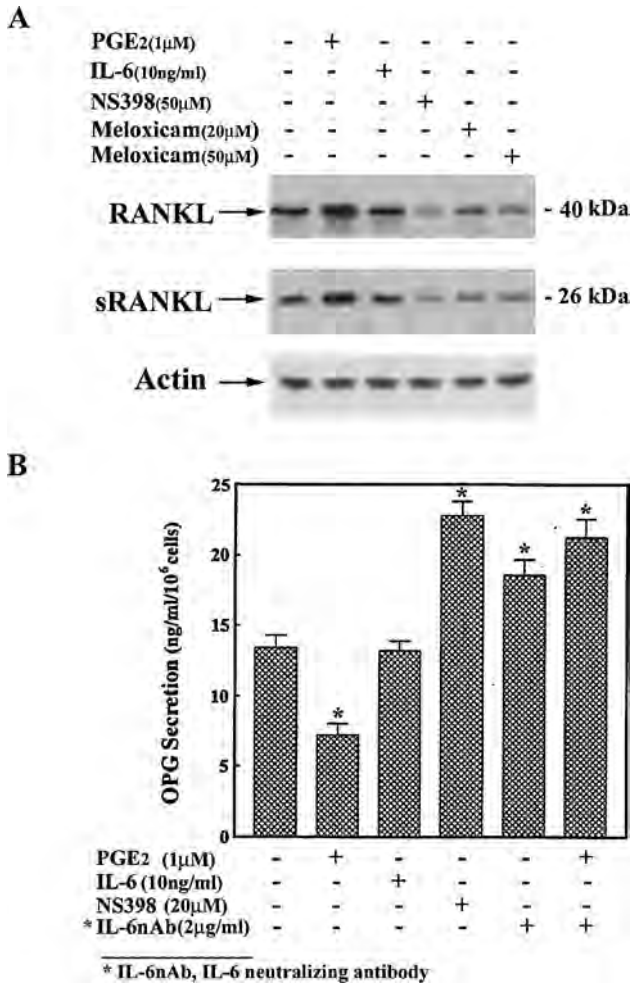


**FIGURE 2.** Effects of PGE2 and IL-6 on RANKL/OPG secretion and RANK expression. (**A**) *Effects of PGE2 and IL-6 on RANKL/OPG production by osteoblasts*. Cultured cells were treated with vehicle or various factor or compound as indicated for 2 days. Cell lysates were prepared for the analysis of membrane-bound RANKL, and culture medium was concentrated 100-fold for the analysis of sRANKL by Western blotting (WB). (**B**) *Effects of PGE2 and IL-6 on RANK expression by RAW264.7 cells*. Cells were treated with vehicle or various compounds, as indicated, for 2 days. Protein was extracted and subjected to WB. Data shown are representative of three separate experiments. (**C**) *Effects of PGE2 and IL-6 on OPG protein expression.* MC3T3 cells were cultured in serum free medium and treated with or without various factors or compounds, as indicated, for 2 days (*Upper panel*). Dose-dependent effects of PGE2 and IL-6 on OPG expression (*low panel*). In these experiments, cells were treated with various doses of PGE2 and IL-6, as indicated, for 2 days. After the various treatments, protein was extracted and subjected to Western blot analysis. Equal amounts of total protein (30μg) were loaded in each lane. Data shown are representative of three separate experiments.

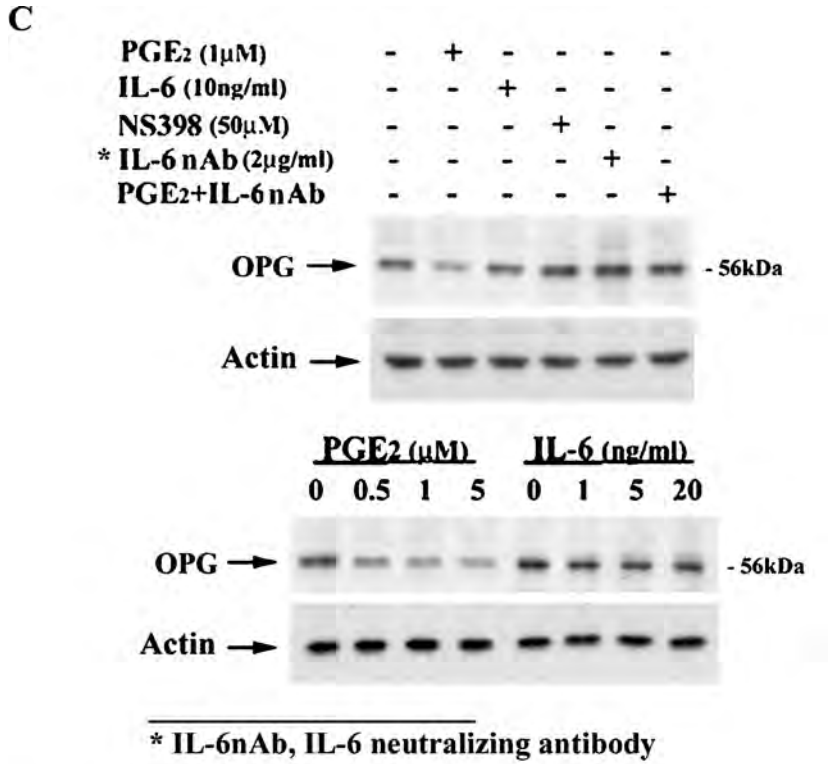


**FIGURE 2.** (**D**) *Effects of PGE2 and IL-6 on RANK expression in osteoclast.* RAW264.7 cells were cultured in serum free medium and treated with vehicle or various factors or compounds, as indicated, for 2 days. After treatment, protein was extracted and subjected to Western blotting. Equal amounts of total protein (30μg) were loaded in each lane. Data shown are representative of three separate experiments.

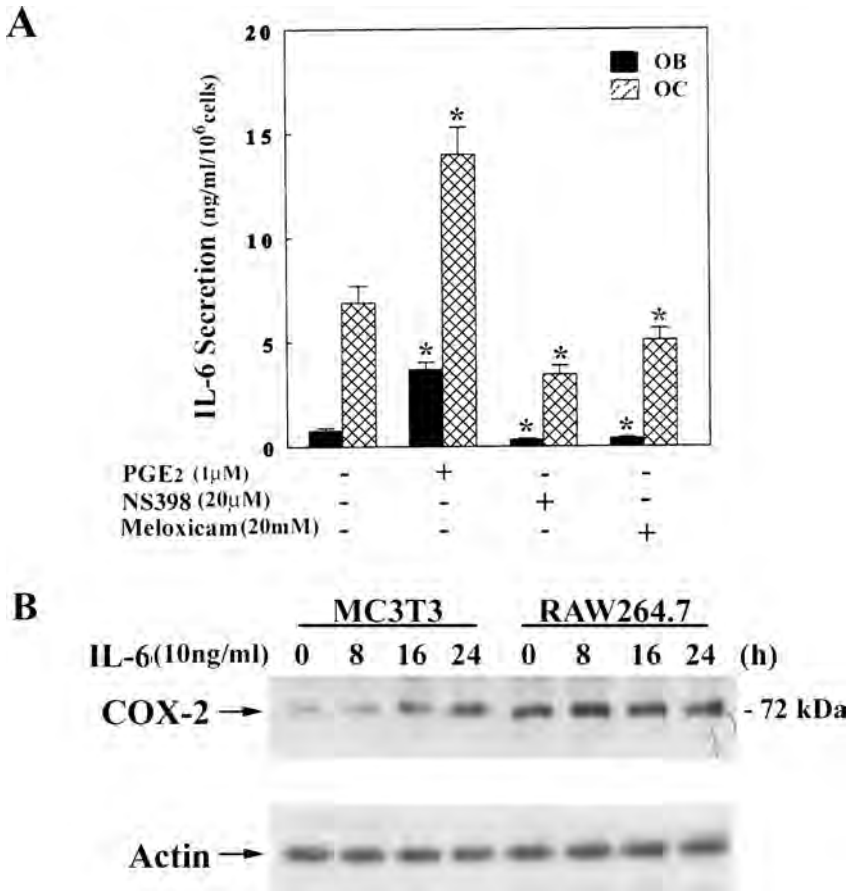


**FIGURE 3.** Reciprocal interactions between the IL-6 and PGE2 systems on bone cells. (**A**) Effects of PGE2 and COX-2 inhibitors on IL-6 secretion. Cultured cells were treated with PGE2, NS398, or meloxicam for 3 days. Medium was collected and subjected to ELISA. (**B**) Effect of IL-6 on COX-2 expression. Cells were treated with 10 ng/ml IL-6 for the times indicated. Protein was extracted and subjected to Western blotting (WB). (**C**) Effect of IL-6 on PGE2 production. Cells were treated with IL-6 for 3 days and ELISA was performed. (**D**) IL-6-induced $EP_4$ and $EP_2$ expression. Cells were treated with 10 ng/ml IL-6 for 2 days. Total RNA was isolated, and subjected to Rt-PCR. (**E**) Protein was extracted and subjected to WB. The results from ELISA were normalized to cell number and represent as mean ± SEM from three separate experiments. Data shown derived from WB and PCR are representative of three separate experiments. $^*P < 0.01$.

the effects of COX-2, PGE2, and IL-6 on the production of RANKL and OPG by MC3T3 cells, as well as RANK expression in RAW264.7 cells. As shown in FIGURE 2A, IL-6 had no detectable effect on the expression of RANKL production, whereas PGE2 increased the expression of both membrane-bound

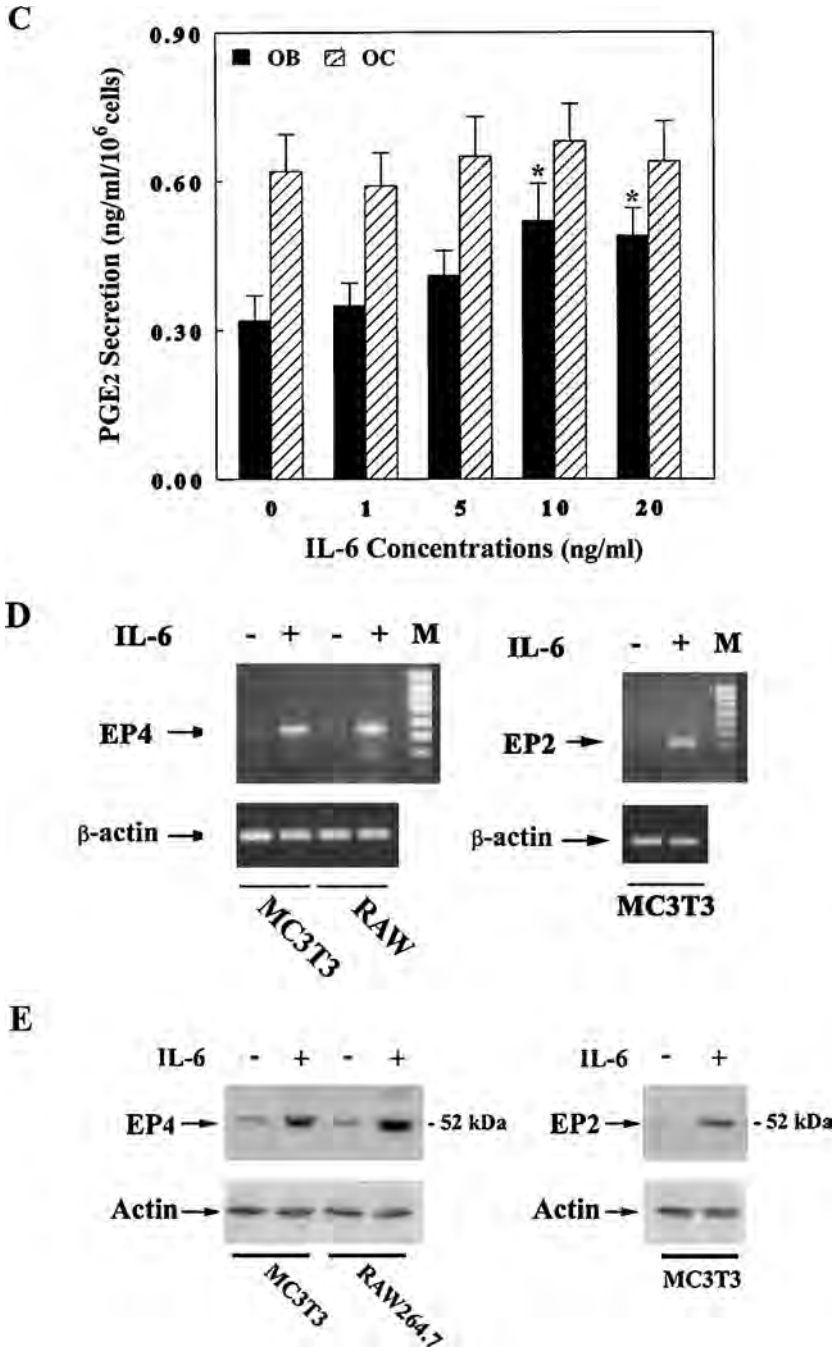


**FIGURE 3.** *Continued*

RANKL and soluble RANKL (sRANKL) levels in MC3T3 cells, while COX-2 inhibitors decreased RANKL expression in these same cells. FIGURE 2B–C demonstrates that PGE2 addition decreases OPG secretion, while COX-2 inhibition significantly stimulates OPG secretion by MC3T3 cells. Although exogenous IL-6 alone had no detectable effect, IL-6-neutralizing antibodies increased OPG secretion and reversed the observed suppression of OPG secretion by PGE2, suggesting that IL-6 mediates the inhibitory effects of PGE2 on OPG production. FIGURE 2D demonstrates that both IL-6 and PGE2 increased the expression of RANK in osteoclast cells. The two distinct COX-2 inhibitors, NS398 and meloxicam, decreased RANK expression in these same cells.

### *Reciprocal Interactions Between the IL-6 and PGE2 Systems*

We next investigated the reciprocal interactions between the IL-6 and PGE2 signaling pathways. Basal IL-6 secretion was 10-fold higher in RAW264.7 cells than in MC3T3 cells. PGE2 significantly stimulated IL-6 production in both cell lines and COX-2 inhibitors inhibited IL-6 secretion (FIG. 3A). RAW264.7 cells expressed a high basal level, but a modest inducible level, of COX-2. Although basal expression was barely detectable, a significant, time-dependent increase in COX-2 expression was observed in MC3T3 cells after IL-6 addition (FIG. 3B). On the other hand, IL-6 had little effect on PGE2 secretion in RAW264.7 cells but significantly increased PGE2 secretion in MC3T3 cells (FIG. 3C). The effect of IL-6 on the expression of EP receptor subtypes in the bone cell lines was also assessed. Rt-PCR revealed that both MC3T3 and RAW264.7 cells express the $EP_4$ receptor subtype mRNA and protein, but do not express detectable levels of $EP_1$, $EP_2$, and $EP_3$ subtype receptors. The expression levels of $EP_4$ in both cell lines were increased by IL-6 (FIG. 3D–E). Although MC3T3 cells had undetectable basal levels of the $EP_2$, the levels were also induced by IL-6.

In the present study, we demonstrate that COX-2 and PGE2 increase both osteoblast proliferation and osteoclast differentiation. These results confirm the observation that disruption of COX-2 gene expression results in defective osteoblast secretion of RANKL and OPG. We also observed that IL-6 stimulation of osteoclast differentiation appears to be due to its effects on PGE2-induced OPG secretion by osteoblasts and upregulation of RANK expression by osteoclasts. In addition, IL-6 increases the expression of both the $EP_4$ and $EP_2$ receptor subtypes in bone cells. All of these events induced by an interaction between the COX-2/PGE2 and IL-6 systems, coordinately, tip the balance of the RANKL/OPG/RANK system in favor of increased osteoclastogenesis and enhanced bone resorption.

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# Androgen-Induced Wnt Signaling in Preosteoblasts Promotes the Growth of MDA-PCa-2b Human Prostate Cancer Cells

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## Abstract

**The high morbidity and mortality associated with prostate cancer (PCa) result from its tendency to metastasize to bone where it produces predominantly osteoblastic lesions. The Wnt signaling pathway plays an important role in embryogenesis, tumorigenesis, osteoblast development, and bone formation. Androgen signaling via the androgen receptor (AR) is critical in both PCa and bone cell growth. We examined the effects of androgens on cell growth and Wnt signaling in the AR-positive MDA-PCa-2b cell line and MC3T3 preosteoblasts, grown alone and in coculture. We show that the potent androgen dihydrotestosterone increases AR expression and transcriptional activity only in the preosteoblasts. Although dihydrotestosterone induced an 80% increase in PCa cell growth when the cells were grown alone, dihydrotestosterone had a more significant effect on MDA-PCa-2b cell proliferation (3.2-fold increase) when the PCa cells were cocultured with preosteoblasts. Dihydrotestosterone addition to preosteoblasts promoted Wnt-dependent transcriptional reporter activity associated with GSK3β$^{S-9}$ phosphorylation and accumulation of nuclear β-catenin as well as elevated Runx2 expression. In addition, the increased proliferation of PCa cells in coculture with MC3T3 cells in response to dihydrotestosterone was abrogated by the addition of either exogenous DKK-1 or sFRP-1 protein, two naturally occurring Wnt antagonists. Finally, we show that the paracrine growth-promoting effect of androgens is limited to MDA-PCa-2b cells. These data imply that Wnt signaling is involved in the androgen-regulated crosstalk between preosteoblasts and PCa cells and suggest that androgens may stimulate growth of some prostate tumor cells indirectly, via up-regulation of Wnt signaling in bone cells.** [Cancer Res 2007;67(12):5747–53]

## Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer deaths in U.S. males. The high morbidity and mortality associated with PCa derive from its tendency to metastasize to bone (1). Although there is evidence that PCa initially induces osteolytic lesions (2), PCa-induced bone metastases are unique in that they typically produce an osteoblastic reaction in bone (3). The precise mechanisms underlying this tendency for PCa cells to target bone and induce an osteoblastic response in the bone microenvironment have not been well delineated. These bony metastases grow more rapidly than primary or other metastatic lesions (3, 4), suggesting that interactions between PCa cells and the bone microenvironment may promote PCa growth and progression.

PCa cell growth in both the primary site and the bone microenvironment is under both autocrine regulation involving androgen-mediated up-regulation of several growth factors (5, 6) and paracrine influences involving the stromal components (4, 7). Clinically, a bidirectional interaction between PCa cells and osteoblasts has been shown to stimulate both bone targeting and bone reaction (4). Several factors, such as basic fibroblast growth factor (4), osteocalcin, bone sialoprotein (8), and interleukin-6 (9), have been shown to be responsible for this pattern of PCa progression. However, the precise events and underlying mechanisms have not been fully elucidated.

The Wnt signaling family includes 19 secreted glycoproteins that have functions related to embryogenesis, cell specification, formation of the body plan, cell growth, differentiation, and apoptosis (10). Wnt signaling plays a central role in osteoblast development and bone formation (11). Wnts promote the lineage commitment of mesenchymal precursor cells and the differentiation of progenitor cell lines into osteoblasts (11, 12). In addition, they stimulate osteoblast maturation and exert direct effects on the formation and turnover of the mature skeleton. Wnts directly stimulate tumor cell growth and survival via autocrine regulation in several types of human cancer, including PCa (13).

Androgen signaling via the androgen receptor (AR) is a key pathway that contributes to PCa progression (14, 15). Androgens also exert direct anabolic effects on normal bone (16, 17). Recent studies show that there is significant crosstalk between the Wnt/β-catenin and AR signaling pathways. Functional colocalization of AR, β-catenin, and Tcf in the nucleus has been reported (18, 19). It has also been shown that β-catenin preferentially binds AR over several other steroid hormone receptors, including the estrogen receptor, progesterone receptor, and glucocorticoid receptor (18). Moreover, forced overexpression of β-catenin augments AR-mediated transcription of several AR-regulated promoters in both prostate and nonprostate cells (19, 20), indicating that β-catenin may act as a coactivator of AR and that AR may require β-catenin to regulate gene expression. Although increasing evidence has shown an interaction between AR and Wnt signaling in PCa cells, the possible crosstalk between the two pathways in the bone microenvironment in the setting of PCa bone metastases has not previously been well delineated.

In the present study, we examined the effects of androgens on cell growth, Wnt signaling activity, and the expression of various components of the Wnt signaling pathway in MDA-PCa-2b, an AR-positive PCa cell line, versus MC3T3, an osteoblastic precursor cell line. Our results reveal an involvement of Wnt signaling in androgen-regulated crosstalk between preosteoblasts and PCa cells

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and indicate that androgens may stimulate MDA-PCa-2b cell proliferation in the bone microenvironment indirectly via modulation of Wnt signaling in the bone cells.

## Materials and Methods

**Cell culture and reagents.** The AR-positive PCa cell line, MDA-PCa-2b, the AR-negative PC-3 cell line, and the MC3T3-E1 osteoblast precursor cell line were obtained from the American Type Tissue Collection. The PC-3ML human PCa cell line, a subline of the PC-3 cell line (21), was a generous gift from Dr. M. Stearns (Department of Pathology, MCP-Hahnemann University, Philadelphia, PA). Cells were cultured in DMEM containing 10% fetal bovine serum. For coculture experiments, MDA-PCa-2b cells were seeded in 12-well cluster culture plates, and MC3T3 cells were seeded in cell-culture inserts with 0.4-μm pores (BD Inc.) as described previously (22). All experiments were done in triplicate and repeated three separate times. Dihydrotestosterone was purchased from Sigma. Recombinant DKK-1 and sFRP-1 proteins were purchased from R&D systems. Enhanced Luciferase Assay kit and β-catenin antibodies were obtained from BD PharMingen. Antibodies against AR, DKK-1, sFRP-1, and β-actin were purchased from Santa Cruz Inc.. Antibodies against GSK3β and phosphor-GSK3β were obtained from Cell Signaling Technology. Runx2 antibodies were purchased from MBL Co.

**Protein isolation and immunoblotting.** Cells cultured under the indicated conditions were lysed, and total protein was isolated as described previously (23). Proteins from the cytosolic and nuclear fractions were isolated using a commercial kit purchased from Pierce, according to the manufacturer's instructions. Protein content was assayed using a kit from Bio-Rad. Western blotting was done as previously described (23). β-actin and histone H1 (for nuclear protein) were used as the internal control in Western blot analyses.

**Transient transfection and luciferase reporter assay.** Transient transfection was done using LipofectAMINE 2000 reagent according to the manufacturer's instruction (Invitrogen). The Tcf luciferase reporter construct pGL3-OT and control vector were generously provided by Dr. B. Vogelstein (John Hopkins Oncology Center, Baltimore, MD). TransLucent AR Reporter Vector and control plasmid were purchased from Panomics Inc. *Wnt3a* expression vector was constructed as described previously (24). Cells were cultured in 12-well cluster plates and transfected with either 1 μg of the reporter plasmid or empty vector as a mock control. Internal normalization was done by cotransfection of the β-galactosidase expression vector (BD ClonTech). After 40 h, the transfected cells were lysed by scraping into reporter buffer (BD ClonTech), total protein concentration was determined, and luciferase and β-galactosidase activities were assayed and quantitated using a TD-20e Luminometer. The resulting activities were normalized to protein concentrations and β-galactosidase activity.

**Immunofluorescence assay.** Cells were incubated on glass coverslips and treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for 24 h. Immunofluorescence assay was done as previously reported (23). Secondary antibodies conjugated to fluorophores were used at a 1:100 dilution and were incubated for 1 h at 37°C followed by 3× 10-min washes. Positively stained cells were evaluated with a fluorescent microscope.

**Alkaline phosphatase assay.** Cultured cells were washed with PBS and sonicated in 10 mmol/L of Tris-HCl buffer (pH, 7.5) containing 0.1% Triton X-100. ALP activity in the lysate was assayed by the hydrolysis of *p*-nitrophenyl phosphate (Sigma) to *p*-nitrophenol. Absorbance was determined at 405 nm and compared with a *p*-nitrophenol (Sigma) standard titration curve. ALP activity was normalized to total protein content.

**Statistical analysis.** All results are given as mean ± SE. The effects induced by the various treatments were compared with untreated control cells using paired Student's *t* test with the Bonferroni adjustment for the comparison of multiple groups. A *P* value of <0.05 was considered significant.

## Results

**Dihydrotestosterone increases MDA-PCa-2b cell proliferation when cocultured with preosteoblasts.** We initially examined the effects of dihydrotestosterone on MDA-PCa-2b cells grown either alone or in coculture with MC3T3 preosteoblasts. As shown in Fig. 1, dihydrotestosterone had a modest, stimulatory effect on MDA-PCa-2b cell growth when cultured alone (80% increase, $P < 0.05$). In contrast, when the PCa cells were cocultured with MC3T3 cells, dihydrotestosterone strikingly increased PCa cell numbers (3.2-fold). Of note, MDA-PCa-2b cell proliferation also increased, even in the absence of dihydrotestosterone, when the PCa cells were cocultured either with MC3T3, a bone-derived fibroblast cell line, or NIH3T3, an embryonic fibroblast cell line, This androgen-independent effect of coculture with stromal cells is likely from the result of secretion of stromal-derived growth factors, as previously reported (4). Because NIH 3T3 cells do not express measurable amounts of androgen receptor, we used these cells in coculture as a negative control for androgenic effects. Our data show that dihydrotestosterone failed to induce a further increase in cell growth when the PCa cells were cocultured with NIH3T3 cells. Dihydrotestosterone had no significant direct or indirect effects on the growth of either MC3T3 or NIH3T3 cells (data not shown).

**Dihydrotestosterone induces AR expression and transcriptional activity in MC3T3 cells.** We next examined AR protein expression in MDA-PCa-2b, MC3T3, and NIH3T3 cells. As shown in Fig. 2*A*, although MC3T3 preosteoblast cells expressed relatively low basal levels of AR, dihydrotestosterone induced a time-dependent increase in AR expression after 24 h. This AR protein induction was significant ($P < 0.01$), with a mean value of 3.2-fold derived from three assays. In contrast, MDA-PCa-2b cells expressed higher basal levels of AR protein, but the expression levels were not further increased by dihydrotestosterone treatment. NIH3T3 cells expressed neither basal nor inducible AR protein. AR

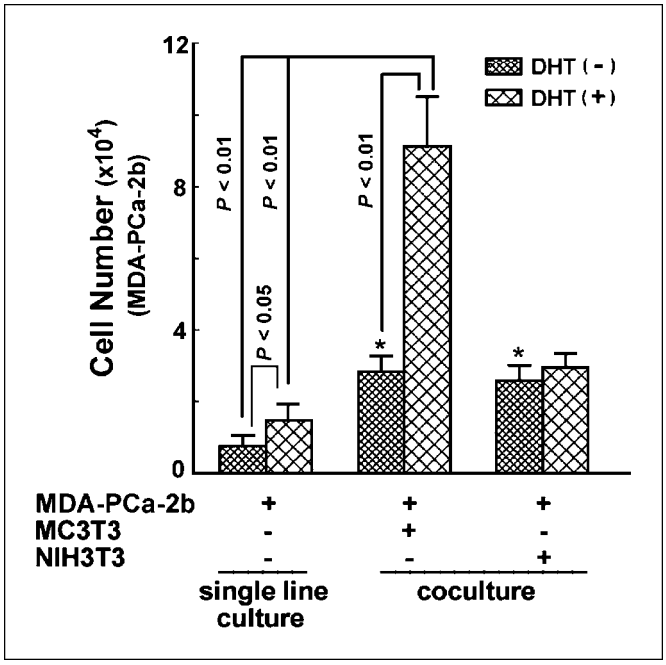


**Figure 1.** Dihydrotestosterone induces the growth of MDA-PCa-2b cells cocultured with MC3T3 osteoblasts. Cells were cultured in serum-free medium and treated with either vehicle or $10^{-8}$ mol/L dihydrotestosterone for 7 d. The number of living cells was counted using a hemacytometer. Data are expressed as means ± SE of three wells from three separate experiments. *, $P < 0.01$ versus the cells cultured alone without dihydrotestosterone treatment. The statistical comparisons among the other groups are shown by *P* value.

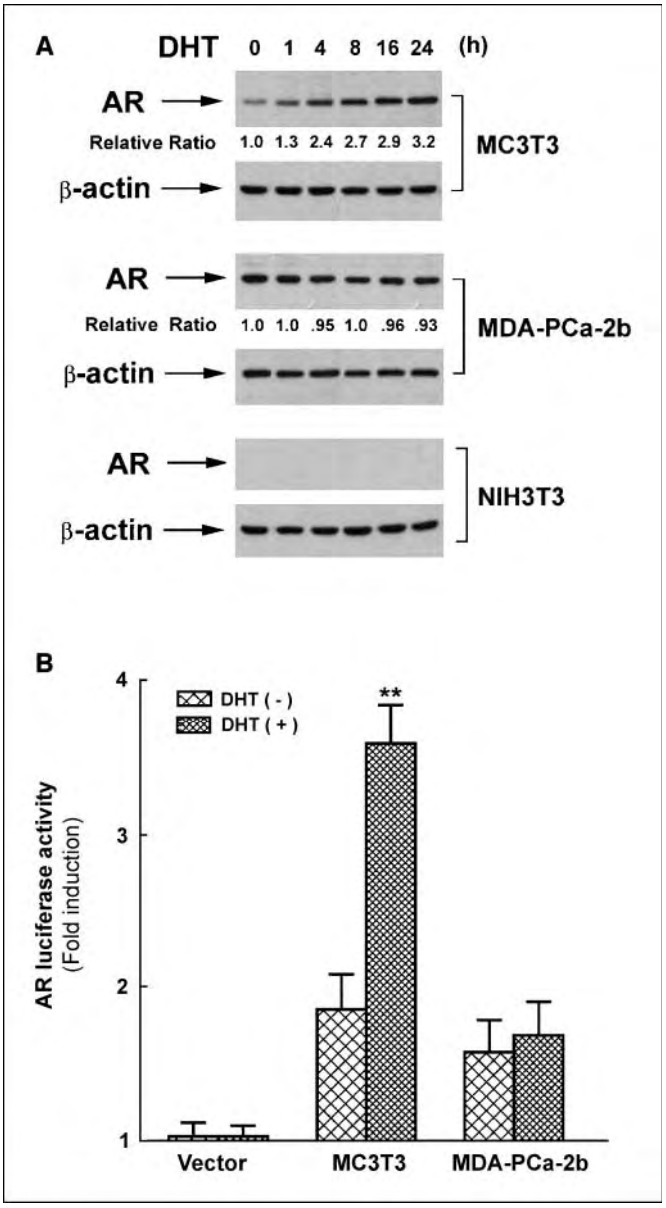


**Figure 2.** Dihydrotestosterone induces AR expression and transcriptional activity in MC3T3 cells. *A,* dihydrotestosterone induces AR protein expression. Cells were treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for the times indicated. Total protein was extracted and subjected to Western blotting. The same blot was stripped and reprobed with β-actin antibody. Normalized quantification (relative ratio of AR to β-actin) of the immunoblots was done by densitometry and is shown at the bottom. Data shown are representative of three separate experiments and expressed as fold induction compared with control. *B,* dihydrotestosterone induces AR transcriptional activity. Cells were cotransfected with the TransLucent AR reporter plasmid (or empty vector as mock control) and the β-galactosidase expression vector and treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for the times indicated. The resulting luciferase activity was normalized to protein concentrations and β-galactosidase activity. Data are expressed as fold induction compared with mock control (100%) and are the mean ± SE from three determinations. **, $P < 0.01$ versus vehicle control.

transcriptional activity was assessed using an AR luciferase reporter assay. As shown in Fig. 2*B*, treatment of MC3T3 cells with dihydrotestosterone enhanced AR luciferase activity, whereas this activity was not influenced by dihydrotestosterone addition in the MDA-PCa-2b cells (Fig. 2*B*). These observations are in agreement with a previous study indicating that the family of MDA-PCa cell lines, including MDA-PCa-2a and -2b, exhibit very low binding affinities for dihydrotestosterone due to mutations in the ligand binding domain of the AR (25).

**Dihydrotestosterone increases Wnt signaling activity in preosteoblasts.** Functional interactions between Wnt and AR signaling in AR-positive cell lines have been shown (18–20). Thus, we tested the effects of dihydrotestosterone on the activation of the Tcf transcription factor using a Tcf-dependent luciferase transcriptional reporter. *Wnt3a*-transfected cells were used as a positive control for Tcf activation in both cell lines. As shown in Fig. 3, dihydrotestosterone addition resulted in a significant enhancement of Tcf-luciferase activity in MC3T3 osteoblast cells, but not in MDA-PCa-2b cells. These data show that androgens induce Wnt activity in MC3T3 preosteoblast cells but not in MDA-PCa-2b cells.

We next examined the effects of dihydrotestosterone on GSK3β protein phosphorylation using Western blot analysis with antibodies specifically against phospho-GSK3β. MC3T3 cells expressed low basal levels of phosphorylated GSK3β protein, and dihydrotestosterone significantly enhanced GSK3β phosphorylation, particularly at $Ser^{9}$, in MC3T3 preosteoblast cells (Fig. 4*A*). This induction was statistically significant ($P < 0.01$), with a mean value of 5.0-fold derived from three assays. There was no effect of dihydrotestosterone on endogenous GSK3β protein levels in these cells. We observed no increased phosphorylation of GSK3β protein in MDA-PCa-2b cells under the same conditions (data not shown).

Activation of the Wnt signaling pathway results in the accumulation of β-catenin in the nucleus associated with heterodimer formation with Tcf and activation of Tcf-dependent transcription. Thus, we examined the effects of dihydrotestosterone on subcellular localization of β-catenin. We also examined the effect

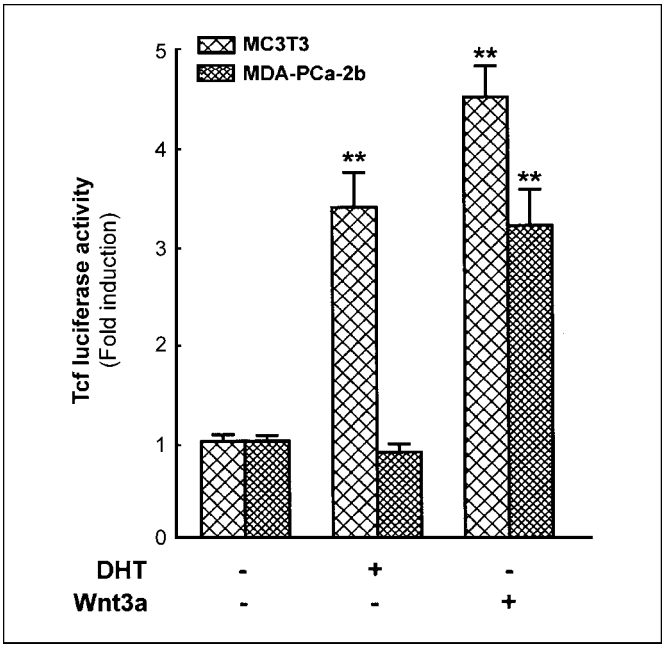


**Figure 3.** Dihydrotestosterone increases Tcf-luciferase activity in osteoblasts. Cells were cotransfected with the Tcf reporter plasmid (or empty vector as mock control) and the β-galactosidase expression vector and treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for 24 h. Wnt3a-transfected cells were used as a positive control. The resulting Tcf-luciferase activities were normalized to protein concentrations and β-galactosidase activity. Data are expressed as fold induction compared with the vehicle control (100%) and represent the means ± SE from three separate determinations. *, $P < 0.05$; **, $P < 0.01$.

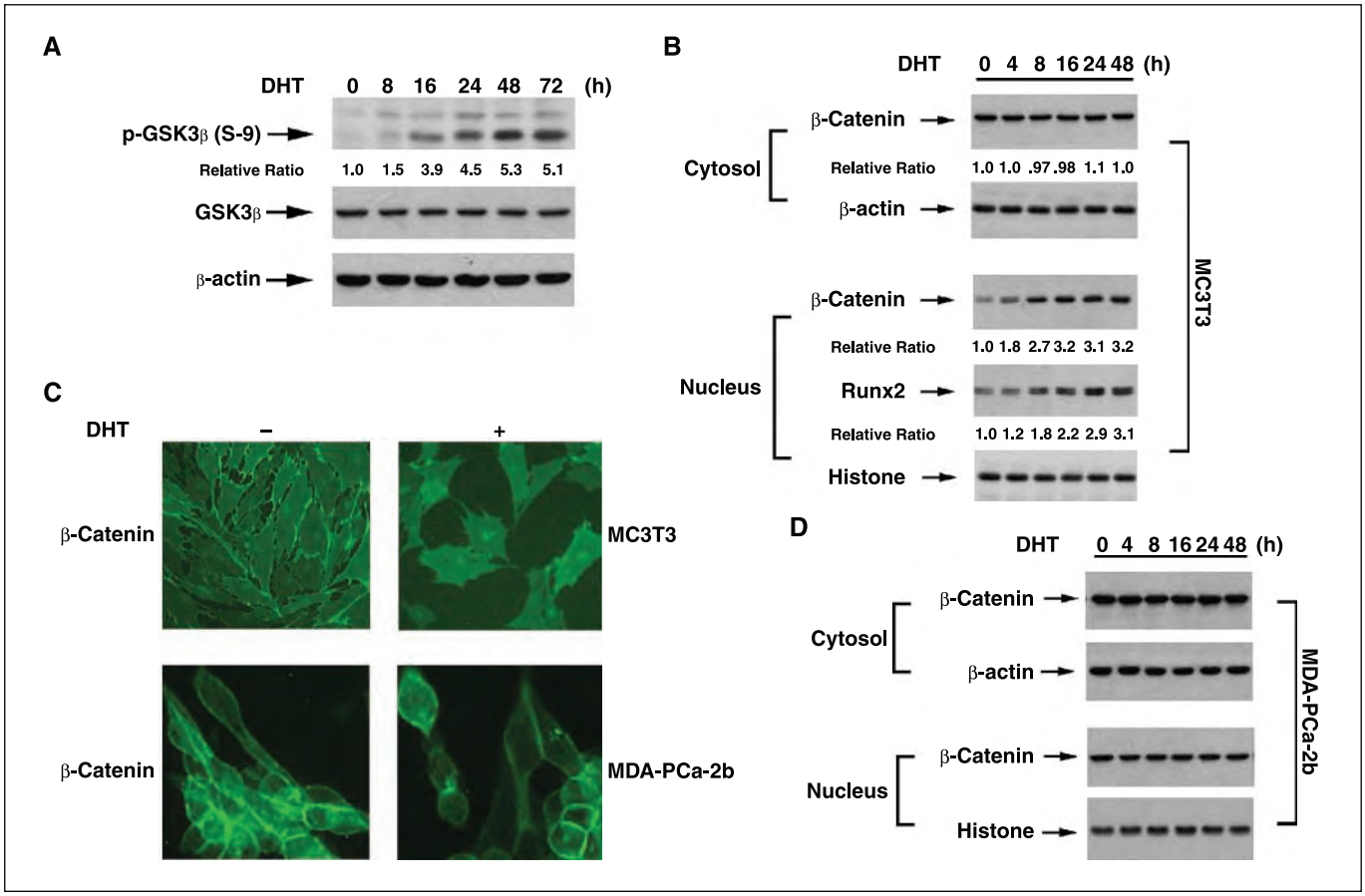


**Figure 4.** Effects of dihydrotestosterone on GSK3β phosphorylation, β-catenin relocalization, and nuclear Runx2 protein levels. MC3T3 cells were treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for the times as indicated. *A,* total protein was extracted and subjected to Western blotting using antibody against phosphorylated (S-9) GSK3β. The same blot was stripped and reprobed with endogenous GSK3β antibody. Normalized quantification (densitometry) of the immunoblots is shown at the bottom as relative ratio (p-GSK3β versus E-GSK3β). Data shown are representative of three separate experiments and expressed as fold induction compared with control. *B,* in separate experiments, protein in the cytosolic or nuclear fractions was extracted and subjected to Western blotting using antibodies against β-catenin or Runx2. The same blot was stripped and reprobed with histone antibodies. Normalized quantification (densitometry) of the immunoblots is shown at the bottom as relative ratio (β-catenin or Runx2 versus histone). Data shown are representative of three separate experiments and expressed as fold induction compared with control. *C,* immunofluorescence assay. Cells were treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L) for 24 h and incubated with anti–β-catenin monoclonal antibodies for 2 h followed by secondary antibodies conjugated to fluorophores for 1 h. *D,* in separate experiments, protein in the cytosolic or nuclear fractions was extracted and subjected to Western blotting using antibodies against β-catenin. The same blot was stripped and reprobed with either β-actin antibodies for cytosolic protein or histone antibodies for nuclear protein. Data shown are representative of three separate experiments.

of dihydrotestosterone on nuclear Runx2 levels in MC3T3 cells because this transcription factor is a direct target gene of the Wnt signaling pathway in bone-related cell lines (26). As shown in Fig. 4*B*, dihydrotestosterone significantly increased both β-catenin and Runx2 nuclear levels in MC3T3 preosteoblasts. The mean values of induction derived from three assays were 3.1- and 3.0-fold ($P < 0.01$), respectively. The dihydrotestosterone effect on β-catenin nuclear localization was confirmed in MC3T3 cells by immunofluorescence assay (Fig. 4*C*). In contrast, dihydrotestosterone had no effect on the subcellular localization of β-catenin in MDA-PCa-2b cells (Fig. 4*D*).

**Inhibition of Wnt signaling suppresses proliferative effects of dihydrotestosterone on PCa cells in coculture with preosteoblasts.** In an effort to establish that dihydrotestosterone enhancement of PCa cell growth in the bone microenvironment was mediated by Wnt signaling pathway activation in preosteoblasts, we determined the effects of DKK-1 and sFRP-1, two natural inhibitors of the Wnt signaling pathway, on PCa growth in this coculture system. As shown in Fig. 5*A*, there was minimal effect of the inhibitors in the presence or absence of dihydrotestosterone on the growth of PCa cells when the cells were grown alone. In coculture with MC3T3, the growth stimulation observed for PCa in the absence of dihydrotestosterone was also largely unaffected by the inhibitors, implying that these effects were Wnt independent. However, both Wnt antagonists inhibited the striking increase in dihydrotestosterone-induced PCa cell growth in coculture (Fig. 5*A*). These findings, together with the results above, indicate that dihydrotestosterone-induced stimulation of MDA-PCa-2B cells in coculture with preosteoblasts is mediated by androgenic effects on Wnt signaling in the bone cells.

We also investigated the effects of dihydrotestosterone specifically on Tcf-luciferase activity, cell proliferation and differentiation in MC3T3 cells in the presence or absence of Wnt inhibitors. As shown in Fig. 5*B*, androgen addition to single cultures of preosteoblasts increased Tcf signaling, which was specifically inhibited by DKK-1 or sFRP-1, establishing that Wnt activation by dihydrotestosterone was responsible. Although we observed little, if any, effects of dihydrotestosterone on proliferation, alkaline phosphatase (ALP) was increased by the addition of the hormone. The inhibitors reduced both basal and dihydrotestosterone-induced

ALP levels. The above results indicated that Wnt antagonists disrupted the effects of dihydrotestosterone-stimulated Wnt activity directly in bone cells and indirectly in MDA-PCa-2b prostate cancer cells.

Both the PC-3 human prostate cancer cell line and its subline, PC-3 ML, were originally derived from human PCa bone metastases and are known to secrete high levels of DKK-1, which might be expected to abrogate the observed growth-promoting effects of coculture with preosteoblasts in the presence of dihydrotestosterone. In fact, we confirmed that androgen addition to cocultures of MC3T3 with either PC-3 or PC-3ML cells did not enhance cancer cell proliferation (Fig. 6*A*). To explore the mechanism underlying the differential effect of dihydrotestosterone in those PCa cell lines, we further examined the expression of Wnt inhibitors in three bone-derived PCa cell lines as well as MC3T3 cells. As shown in Fig. 6*B*, the expression of Wnt inhibitors was barely detectable in MC3T3 bone preosteoblasts and relatively low in MDA-PCa-2b cells. In contrast, PC-3 and PC-3ML cells expressed significantly higher levels of both DKK-1 and sFRP-1. Thus, high basal expression levels of these potent Wnt inhibitors in PC-3 and PC-3ML cells may explain the relative lack of effect of coculture with preosteoblasts in the presence or absence of dihydrotestosterone in these particular PCa cell lines.

## Discussion

Prostate cancer (PCa) is unique in that it retains some degree of androgen sensitivity, even in its late stages. Another distinguishing feature of PCa is its tendency to metastasize to bone (1, 3) and to induce an osteoblastic reaction in the bone microenvironment (2, 3). The high morbidity and mortality associated with this disease are directly related to these bone metastases (3). Bone lesions induced by PCa cells grow at a more rapid rate than primary PCa or metastatic PCa in other sites (3, 4), suggesting that bone stromal cells provide a particularly favorable soil for PCa cell growth and progression (27) by secreting soluble growth factors (4, 28, 29). Androgens have been shown to influence normal and abnormal prostate epithelial cell growth both directly (5, 6) and,

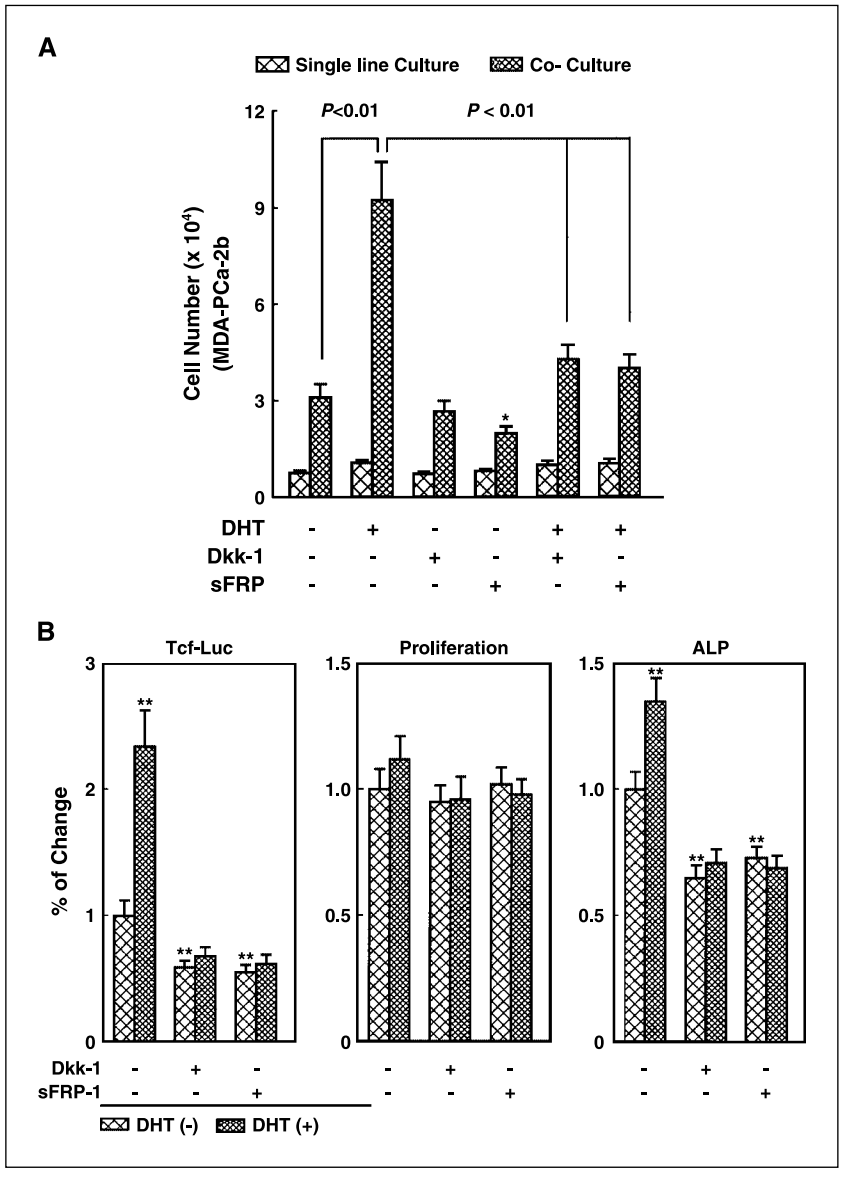


**Figure 5.** Inhibition of Wnt signaling suppresses the growth-promoting effects of dihydrotestosterone. *A,* effect of Wnt signaling inhibition on dihydrotestosterone-induced PCa cell proliferation. Cells were seeded for single line culture and coculture as described previously and subjected to the following treatments: vehicle; or $10^{-8}$ mol/L dihydrotestosterone, or recombinant DKK-1 (1 μg/mL), or sFRP-1 (10 μg/mL), either alone or in combination with dihydrotestosterone ($10^{-8}$ mol/L), as indicated, for 7 d. Number of living cells was counted using a hemacytometer. Data are the means ± SE of three wells from triplicate determinations. *, $P < 0.05$ versus vehicle control. The statistical comparisons among the other groups are shown by the *P* value. *B,* effect of Wnt signaling inhibition on dihydrotestosterone effects on preosteoblasts. Cultured MC3T3 cells were treated with either vehicle or dihydrotestosterone ($10^{-8}$ mol/L), or DKK-1 (1 μg/mL), or sFRP-1 (10 μg/mL) for 24 h. The luciferase reporter activity, proliferative rate, and ALP activity were assayed in separate experiments. Data represent the means ± SE from three separate determinations. *, $P < 0.05$; **, $P < 0.01$.

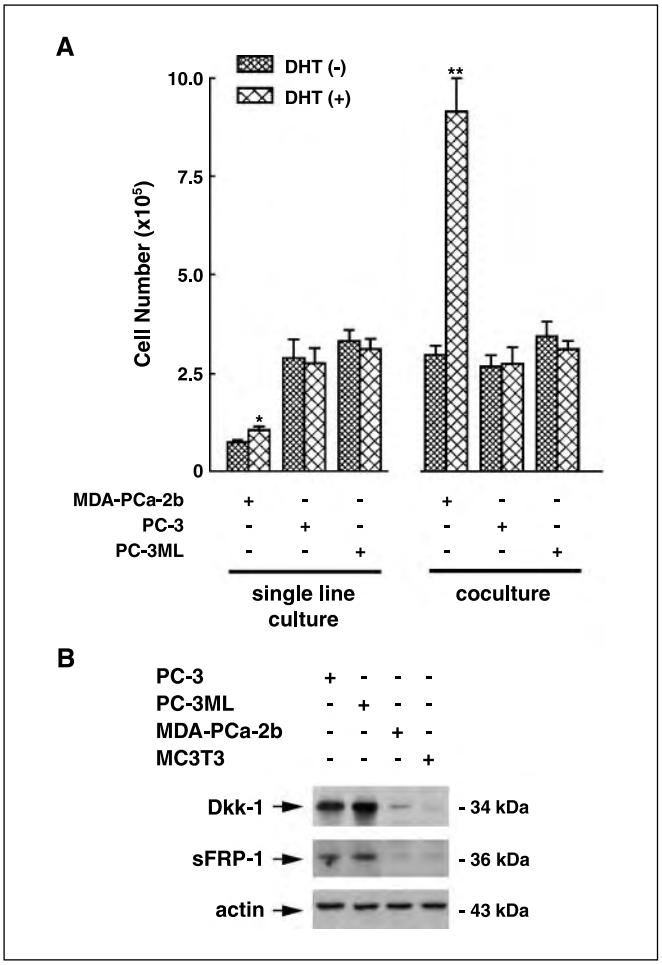


**Figure 6.** Dihydrotestosterone effects on PCa growth in coculture are limited to the MDA-PCa-2b cell line. *A,* effects of dihydrotestosterone on the growth of PC-3 and PC-3ML cell lines. Cells were cultured in serum-free medium either alone or cocultured with MC3T3 cells in the presence or absence of $10^{-8}$ mol/L dihydrotestosterone for 7 d. The number of living cells (PC-3 or PC-3ML cells) was counted using a hemacytometer. Data are expressed as means ± SE of three wells from three separate experiments. *, $P < 0.05$; **, $P < 0.01$ versus control. *B,* protein expression of DKK-1 and sFRP-1. Total protein was isolated and subjected to immunoblotting using antibodies against DKK-1 or sFRP and reprobed with β-actin antibody for internal control. Data shown are representative of three separate experiments.

even more strikingly, indirectly, via modulation of stromal growth factor expression and secretion (4, 7). Clinically, bidirectional interactions between PCa cells and osteoblasts stimulate both the PCa cell growth in bone and the accompanying osteoblastic reaction (4, 30). However, the precise molecular events and the role of androgens in the vicious cycle of PCa/bone cell crosstalk have not been well elucidated.

We used an *in vitro* coculture system to investigate the indirect effects of androgens on PCa cell growth via interactions with preosteoblasts. We selected the MDA-PCa-2b cell line for these studies because (*a*) they are derived from a human PCa bone metastasis, (*b*) they express AR protein but have an impaired response to androgens (31), and (*c*) they produce an osteoblastic bone reaction *in vivo* (32). The MC3T3 preosteoblast cell line used in the cocultures, in contrast, expresses functional AR protein. Although this *in vitro* system does not mimic the full *in vivo* bone microenvironment that contains endothelial cells, osteoclasts, and bone matrix, it enabled us to focus specifically on androgenic modulation of preosteoblast-derived growth factors that stimulate PCa growth.

Androgens play a critical role in both PCa development and progression (14, 15). It has been shown that androgens activate growth-promoting, growth-inhibitory, and/or cell differentiation pathways directly in AR-expressing PCa cells, and the balance of these activities may depend on the stage of differentiation of the cancer cells (33). Androgens also target bone osteoblasts and osteoblast precursors, leading to enhanced osteoblast differentiation and bone formation (16, 17). In single cultures of preosteoblast cells, dihydrotestosterone addition induced the expression of AR protein and activated AR signaling, as measured by AR luciferase activity. This activation of AR signaling in preosteoblast cells was accompanied by evidence of activated canonical Wnt signaling and increased differentiation, as measured by ALP production. Moreover, addition of Wnt inhibitors, DKK-1 and sFRP-1, abrogated the effects of dihydrotestosterone both on Wnt signaling and ALP production in the preosteoblast cells. In contrast, dihydrotestosterone addition to single cultures of MDA-PCa-2b cells had no demonstrable effects on either AR expression or Wnt signaling and resulted in a modest enhancement of cellular proliferation. In contrast, the stimulatory effect of preosteoblasts on MDA-PCa-2b cell proliferation in coculture was markedly enhanced by the addition of androgens (dihydrotestosterone). Furthermore, the addition of Wnt inhibitors to the cocultures abrogated the effect of dihydrotestosterone-stimulated preosteoblasts on MDA-PCa-2b proliferation. Our results strongly imply that androgenic activation of Wnt signaling in preosteoblasts increases the secretion of PCa growth-promoting soluble factors in the cocultures.

We provide evidence that the paracrine growth-promoting effects of androgen-stimulated preosteoblasts on MDA-PCa-2b proliferation are mediated by Wnt signaling in the bone cells. This conclusion is supported by the lack of effect of dihydrotestosterone-stimulated preosteoblasts on the proliferation of PC-3 or PC-3ML cells in coculture because both of these PCa cell lines secrete high levels of Wnt inhibitors. Interestingly, the MDA-PCa-2b cell line is the only one of these three bone-derived PCa cell lines that produces an osteoblastic bone reaction *in vivo* (32). We postulate that the high basal levels of both Wnt inhibitors (DKK-1 and sFRP) by the PC-3 and PC-3 ML cell lines prevent the effect of androgens on Wnt signaling in the preosteoblasts in coculture.

Although many previous reports have shown interactions between the AR and Wnt signaling pathways in PCa cells (13, 34, 35), this is the first report to show such interactions in preosteoblasts. Previous investigators, using prostate and colon cancer cells, as well as neuronal cells, concluded that there is negative cooperativity between AR and Wnt signaling, i.e., AR-mediated repression of β-catenin/Tcf gene activation (19, 36, 37). It is of note, therefore, that our data show a stimulatory effect of androgens and AR on canonical Wnt signaling in bone preosteoblasts.

Our findings are thus consistent with a mechanism underlying the predilection for PCa cells to metastasize to bone and induce osteoblastic activity. Androgens regulate normal prostate development and are required for prostate tumorigenesis. In both development and carcinogenesis, many of the androgenic effects on prostate epithelial cells are elicited via signaling through stromal AR (38–40). We confirmed that PCa growth in the bone is stimulated by androgenic effects on stromal cells and specifically by activation of Wnt signaling in preosteoblasts. This androgen-mediated Wnt activation in preosteoblasts leads to the secretion of

soluble factors that stimulate the proliferation of a bone-derived human PCa cell line, MDA-PCa-2b. Once these factors are identified, we will determine whether they also enhance non–bone-derived human prostate cancer cell proliferation. This may have important clinical relevance in the management of PCa. Our data predict that treatment with either androgen-ablative or Wnt-inhibitory therapy early in the course of the disease (before bone metastases) may inhibit both the development of bone metastases and the accompanying osteoblast reaction. It is clear from the current literature that targeting cancer cells directly, even with multimodality approaches, often leads to resistance and relapse. Therapies aimed at the noncancerous niche, i.e., bone and vasculature, may produce more long-lasting disease control.

## Acknowledgments

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# Androgens Promote Preosteoblast Differentiation via Activation of the Canonical Wnt Signaling Pathway

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**ABSTRACT: Although androgens stimulate bone formation the precise events underlying these effects have not been elucidated. Wnt signaling plays a central role in osteoblast development and bone formation. We demonstrated that dihydrotestosterone (DHT) significantly stimulates MC3T3 preosteoblast differentiation with no effect on cell growth. This effect of DHT was accompanied by increased Wnt signaling in the same cells. Moreover, the stimulatory effects of DHT on preosteoblast differentiation were inhibited by overexpression of soluble frizzed-related protein (sFRP), a naturally occurring Wnt antagonist. These results suggest that androgens promote preosteoblastic differentiation via effects on the canonical Wnt signaling pathway.**

**KEYWORDS: androgens; preosteoblasts; Wnt signaling**

## INTRODUCTION

Androgens play a critical role in the development and maintenance of the skeleton in both sexes. Androgen receptors (AR) are detected in a variety of bone cells, including osteoblasts, osteocytes, and bone marrow stromal cells.[1] Studies using nonaromatizable androgens in ovariectomized rats demonstrate direct actions of androgens on the preservation of bone mass.[2] These reports suggest that androgens, in contrast to estrogens, have little effect on the inhibition of bone resorption but, rather, directly stimulate bone formation. However, the precise mechanisms underlying the observed anabolic effects of androgens on bone cells are unclear. Moreover, several *in vitro* studies have yielded conflicting and contradictory data. Androgens have been reported to enhance both the proliferation and differentiation of primary cultures of osteoblast-like cells.[3] However, that same report demonstrated a direct inhibitory effect of

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DHT on a human fetal osteoblast cell line *in vitro*.[3] Davey and Morris also reported that androgens can inhibit osteoblast development in the absence of estrogen and in states of low bone turnover.[4] The reported discordant effects of androgens on bone cells warranted further investigation.

The canonical Wnt signaling pathway plays a central role in bone mesenchymal cell lineage commitment, promoting osteoblastic differentiation and stimulating bone formation. While various bone-related cells, including osteoblasts, express androgen receptor (AR) protein, the cross talk between the AR and Wnt signaling pathways in bone cells has not previously been investigated.

This study demonstrates that although the nonaromatizable androgen, dihydrotestosterone (DHT), has no effect on preosteoblastic cell growth, androgens significantly promote preosteoblast differentiation. This effect of DHT is accompanied by activation of canonical Wnt signaling, as evidenced by increases in Wnt-dependent transcriptional reporter activity, enhanced GSK3$\beta^{S-9}$ phosphorylation, accumulation of nuclear β-catenin and increased nuclear Runx2 expression. Moreover, this effect of DHT can be prevented in the same cell line by forced overexpression of soluble frizzed-related protein (sFRP)-1, a natural inhibitor of the Wnt signaling pathway. These results suggest that androgen-promoted preosteoblast differentiation is mediated, at least in part, by activation of canonical Wnt signaling.

## MATERIALS AND METHODS

### *Cell Culture and Reagents*

The MC3T3-E1 cell line was obtained from the American Type Tissue Collection (ATCC). DHT was purchased from Sigma (St Louis, MO, USA). Enhanced Luciferase Assay kit was obtained from BD Pharmingen (San Diego, CA, USA). Antibodies against AR were purchased from Santa Cruz BioTech, Inc. (Santa Cruz, CA, USA). Antibodies against GSK3β, phosphor-GSK3$\beta^{ser-9}$ and phosphor Akt$^{ser-473}$ were obtained from Cell Signaling Tech. (Beverly, MA, USA). β-catenin antibodies were obtained from BD Pharmingen Inc. (San Diego, CA, USA). Runx2 antibodies were purchased from MBL International Co. (Woburn, MA, USA).

### *Alkaline Phosphatase Activity (ALP) Assay*

ALP activity was measured in cell lysates and by staining of cultured cells using an ALP assay kit and ALP staining kit, respectively (Sigma), according to the manufacturer's instructions.

### *Protein Isolation, Immunoblotting, and Immunofluorescence Assay*

Total protein isolation and immunoblotting were performed as described previously.[5] Proteins from the cytosolic and nuclear fractions were isolated using a commercial kit purchased from PIERCE (Rockford, IL, USA), according to the manufacturer's instructions. Immunofluorescence assay was performed as previously reported.[6]

### *Transient Transfection and Luciferase Reporter Assay*

The generation of the TCF luciferase reporter construct pGL3-OT and control vector were described previously.[6] TransLucent AR Reporter Vector and control plasmid were purchased from Panomics Inc. (Redwood City, CA, USA). The methodology for luciferase reporter assay and transient transfection were carried out as previously described.[6]

## RESULTS

### *Effect of DHT on AR Expression and AR Luciferase Activity in MC3T3 Preosteoblasts*

We initially examined AR protein expression in MC3T3 and NIH3T3 cells (used as a negative control). As shown in FIGURE 1A, MC3T3 cells expressed relatively low basal levels of AR protein and DHT increased AR expression after 24 h. This AR protein induction was significant ($P < 0.01$) with a mean 3.2-fold increase after DHT addition (three separate assays). In contrast, NIH3T3 fibroblast cells expressed neither basal nor inducible AR protein. AR transcriptional activity was next assessed. As shown in FIGURE 1B, treatment of MC3T3 cells with DHT enhanced AR luciferase activity in a time-dependent manner.

### *DHT-Induced Tcf Transcriptional Activity in MC3T3 Preosteoblasts*

We tested the effects of DHT on activation of the Tcf transcription factor using a Tcf-dependent luciferase transcriptional reporter. As shown in FIGURE 2, DHT addition resulted in a significant enhancement of Tcf-luciferase activity in MC3T3 cells. This induction was both time- and dose-dependent.

### *DHT Promoted Akt and GSK3β Phosphorylation*

The activation of GSK3, a critical component in the Wnt pathway, is regulated by Akt activity.[7] Activated Akt by phosphorylation at the site of serine$^{473}$

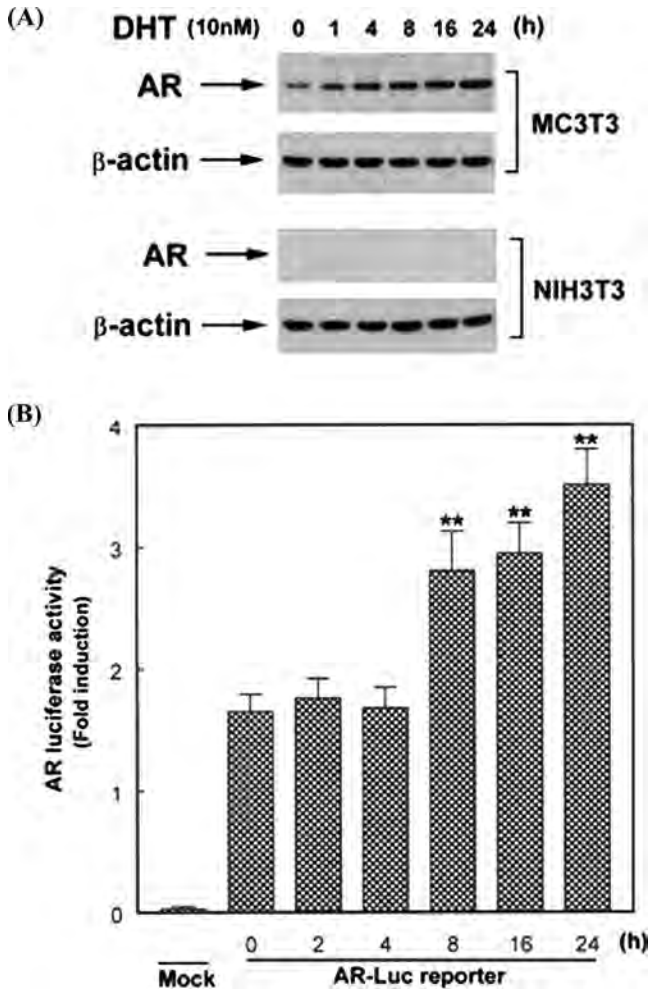


**FIGURE 1.** Effects of DHT on AR expression and AR luciferase activity in MC3T3 preosteoblast cells. (**A**) DHT increases AR protein expression. MC3T3 cell lysates were prepared and subjected to Western blotting. (**B**) DHT induces AR transcriptional activity. Data are the mean ± SE from three determinations.* $P < 0.05$, and** $P < 0.01$ versus vehicle control.

promotes GSK3$\alpha^{ser-21}$ and GSK3$\beta^{ser-9}$ phosphorylation, thereby inhibiting both GSK3β and GSK3α activity.[8] We next examined the effects of DHT on Akt and GSK3α/β protein phosphorylation. Although MC3T3 cells expressed low basal levels of phosphorylated Akt and GSK3α/β protein, DHT significantly enhanced Akt$^{ser473}$ and GSK3β phosphorylation, particularly at serine 9, in MC3T3 cells (FIG. 3A, B). There was no effect of DHT on endogenous

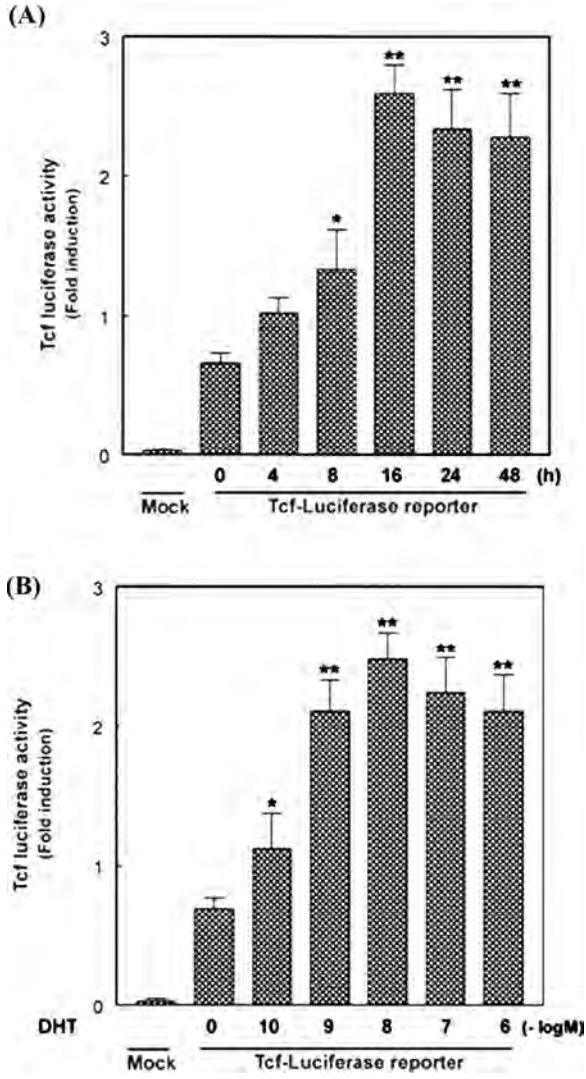


**FIGURE 2.** DHT-induced Tcf transcriptional activity in MC3T3 preosteoblast cells. (**A**) Time course of DHT ($10^{-8}$ M) induction. (**B**) Dose-response of DHT effects. Results represent the mean ± SE from three determinations.* $P < 0.05$, and** $P < 0.01$ versus vehicle control.

Akt and GSK3 protein levels in these cells. These data demonstrate an association between DHT-induced $Akt^{ser473}$ phosphorylation and GSK3β activity, which may be an important component of canonical Wnt signaling activation in preosteoblasts.

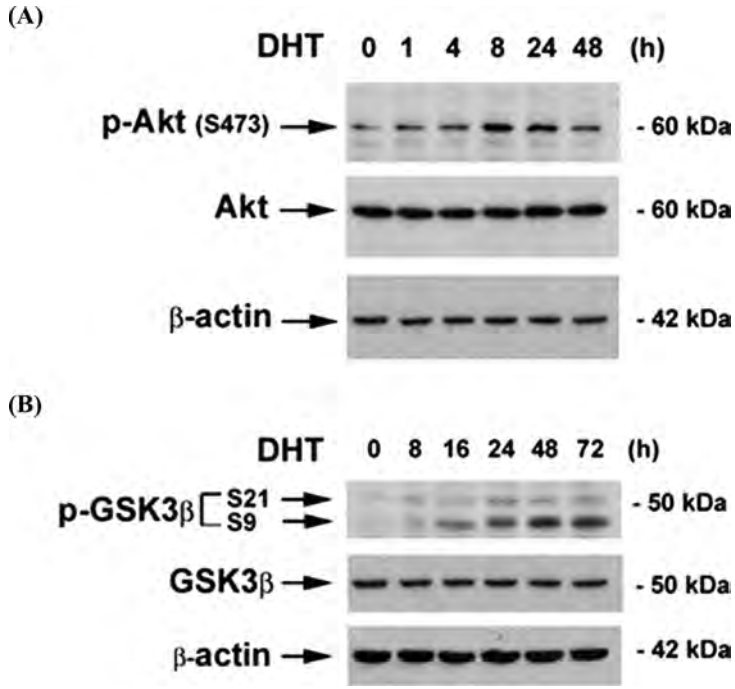


**FIGURE 3.** DHT promotes both Akt and GSK3β phosphorylation in MC3T3 preosteoblast cells. MC3T3 cell lysates were prepared after treatment with $10^{-8}$ M DHT, and subjected to Western blotting. Data shown are representative of three separate experiments.

### *Effect of DHT on the Nuclear Levels of β-Catenin and Runx2*

Activation of the canonical Wnt signaling pathway results in accumulation of β-catenin in the nucleus, heterodimer formation with Tcf, and activation of Tcf-dependent transcription. Thus, we examined the effects of DHT on the subcellular localization of β-catenin and nuclear Runx2 levels (a direct target gene of the Wnt signaling in bone-related cell lines)[9] in MC3T3 preosteoblast cells. As shown in FIGURE 4A, DHT significantly increased both β-catenin and Runx2 nuclear protein levels. The DHT effect on β-catenin nuclear localization was confirmed in MC3T3 cells by immunofluorescence assay (FIG. 4B).

### *Inhibition of Wnt Signaling Abrogated DHT Effects on MC3T3 Preosteoblast Cells*

To determine whether DHT-induced Wnt signaling activation is critical to its biological activity in preosteoblasts, we established an MC3T3 subline that stably overexpresses sFRP, a naturally occurring Wnt inhibitor (sFRP-subline). The effects of DHT on cell proliferation and differentiation were

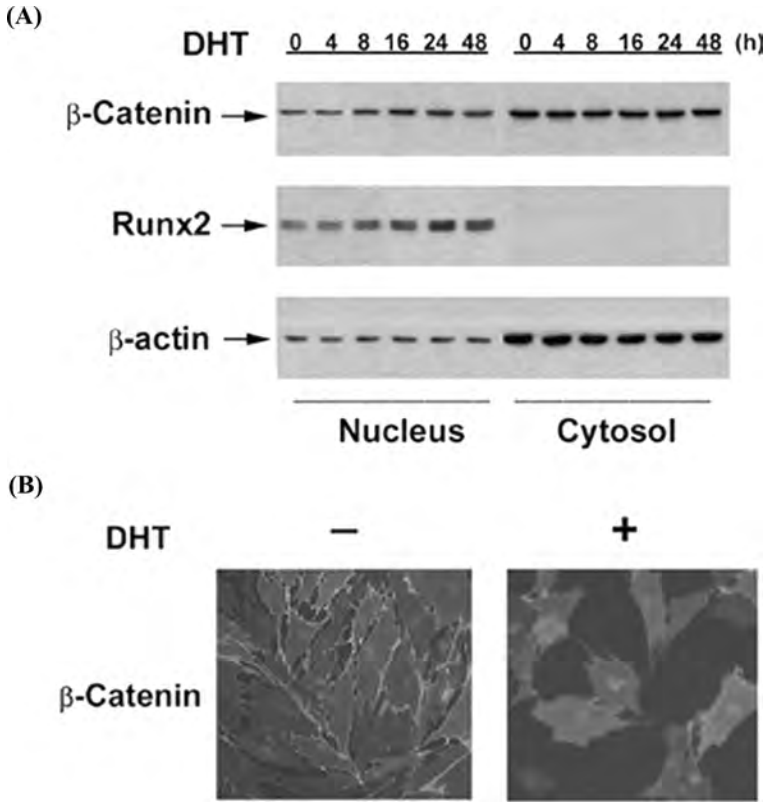


**FIGURE 4.** Effect of DHT on the nuclear levels of β-catenin and Runx2 in MC3T3 preosteoblast cells. (**A**) Western blot. The cytosolic and nuclear proteins were isolated from MC3T3 cells, and subjected to Western blotting. (**B**) Immunofluorescence assay. MC3T3 cells were treated with either vehicle or DHT ($10^{-8}$M) for 24 h and subjected to immunofluorescence stain. Data shown are representative of three separate experiments.

examined and compared between the two MC3T3 cell lines. As shown in FIGURE 5A, DHT did not influence parental MC3T3 cell proliferation, and had a moderate inhibitory effect on the growth of the sFRP-subline. However, DHT significantly stimulated parental MC3T3 differentiation as determined by ALP assay and ALP staining (FIG. 5B, C). In contrast, the MC3T3-sFRP cells exhibited lower basal ALP expression and this expression was not increased by DHT addition.

## SUMMARY

Our data indicate that the potent, nonaromatizable androgen dihydro-13testosterone (DHT) promotes the differentiation of the MC3T3 preosteoblast

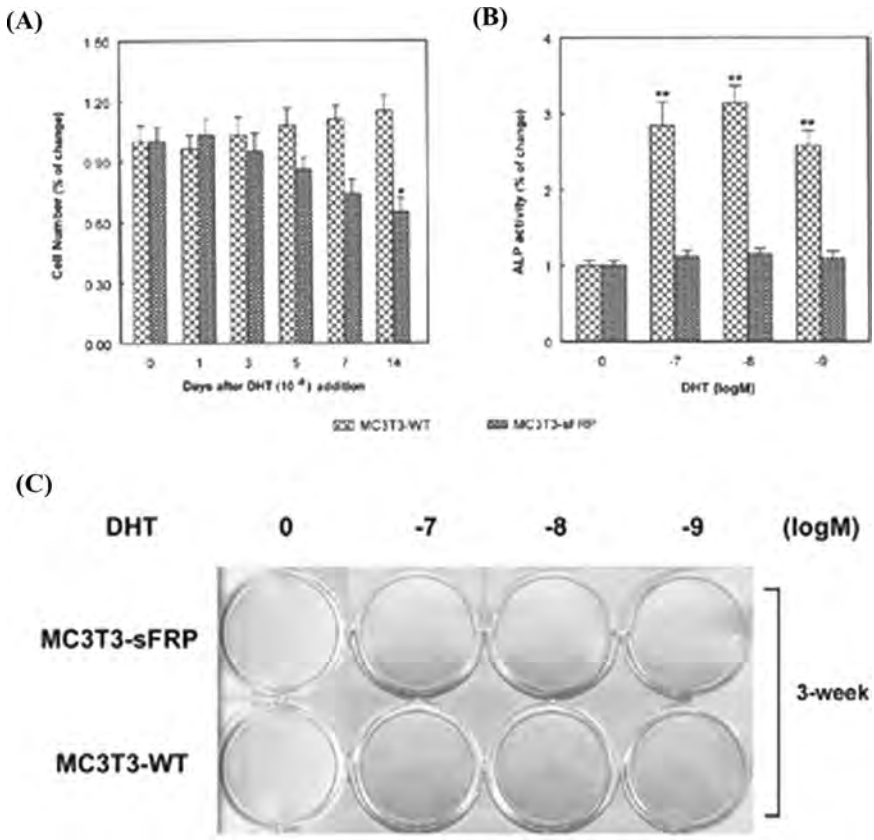


**FIGURE 5.** Inhibition of Wnt signaling mitigated DHT effects on MC3T3 preosteoblast cell differentiation. (**A**) Cells were treated with DHT for 7days and cell numbers were counted using a hemacytometer. (**B**) Effect of DHT on alkaline phosphatase (ALP) activity. MC3T3 cells were treated with $10^{-8}$ M DHT for 21 days, and ALP activity was assayed in cell lysates. Data shown (A&B) are means ± SE of three independent assays. (**C**) Both cell lines were exposed to varying levels of DHT for 3 weeks and stained for ALP activity.

cell line, as determined by alkaline phosphatase expression. In addition, DHT activated canonical Wnt signaling in these same cells, and this effect appears to be initiated by activation of Akt followed by phosphorylation and inactivation of GSK-3β. Finally, forced overexpression of the Wnt inhibitor sFRP blocked the effect of DHT on alkaline phosphatase expression. These results indicate that androgens promote preosteoblast cell differentiation and that this effect is mediated, at least in part, by activation of the canonical Wnt signaling pathway.

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